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Antimicrobial and Resistance-Modifying Activities of LY2183240 Regioisomers

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ABSTRACT

The rapid development of antimicrobial resistance over the past three decades represents a critical public health threat and urgent challenge.

In this context, in order to establish lead compounds or identify novel modes of action one approach is to re-investigate drugs that affect eukaryotic processes for antimicrobial activity, particularly if their drug targets have homologies with bacterial proteins. In this study, regioisomers of LY2183240, a potent inhibitor of anandamide transport and fatty acid amide hydrolase were selected after a small screening study.

The 2,5-LY2183240 regioisomer was shown to possess potent antimicrobial activity selective towards certain Gram-positive bacteria, which included *Staphylococcus aureus* and *Bacillus subtilis*, but not *Enterococcus faecalis* or *Streptococcus pneumoniae*. Conversely, the 1,5-LY2183240-regioisomer had no anti-bacterial activity strongly implicating the position of the carbamoyl on the tetrazole in the structure-activity relationship of the molecule.

Investigation of the mechanism of antimicrobial activity suggested that while 2,5-LY2183240 had bacteriostatic activity this was probably not due to inhibition of protein or teichoic acid synthesis. Nevertheless, this activity may be related to the inhibition of bacterial fatty acid synthesis. Supporting this hypothesis, addition of exogenous fatty acids within Tween 80 was able to compromise 2-5-LY2183240 anti-staphylococcal activity. Based on the spectrum of activity and known redundancies at each of the steps in the fatty acid synthesis pathway, the most likely target was deduced to be FabI. However, characterization of a 2,5-LY2183240-resistant mutant revealed no alteration to the deduced amino acid sequence of FabI or relevant changes to FabI protein expression, an observation confirmed by analysis of the *fabI* promoter region and western blot studies. Since resistance to 2,5-LY2183240 could be mediated through non-target related factors such as drug efflux or drug entry into the cell, FabI could not be ruled out as a potential target. In addition, whole cell protein profiling revealed the 2,5-LY2183240-mutant to have differences in the expressions of several proteins, suggesting resistance may occur through a more global effect. Due to the promiscuous nature of LY2183240, the

possibility of having multiple targets in *S. aureus* that collectively exhibit a bacteriostatic effect cannot be ruled out.

This study also showed that the LY2183240 regioisomers are specific inhibitors of class C β-lactamases with optimum inhibitory activity dependent upon the position of the carbonyl on the tetrazole heterocyclic group (K_i values of 1.8 and 2.45 μM for 1,5- and 2,5-LY2183240, respectively). Molecular modelling suggested that LY2183240 regioisomers bind within the catalytic site of class C β-lactamases, interacting with some of the same residues, including Tyr^{150}, Lys^{315} and Thr^{316}, used by the substrate nitrocefin and β-lactamase inhibitors. The results substantiate the competitive inhibition model determined by enzyme kinetic studies. Furthermore, mass spectrometry data revealed non-covalent interactions between AmpC β-lactamase and LY2183240 regioisomers, which together with the non-reversible inhibitory activity, may indicate that both regioisomers present a high affinity towards this cephalosporinase and dissociate very slowly from the enzyme. LY2183240 may prove useful as a chemical scaffold for the development of novel and highly selective inhibitors of class C β-lactamases.

IMPACT STATEMENT

Antimicrobial resistance is a fluid and constantly evolving challenge. The dimension of the problem worldwide and the influence of antimicrobial resistance on public health, and on costs to the health-care sector and social impact are still largely unknown. With the relative absence of new antimicrobials coming to market and with new threats arising from either Gram-positive or Gram-negative bacteria, the number of drug options is worryingly close to none or only a single effective agent for some life-threatening infections. In addition, several of β-lactam-degrading enzymes are rapidly disabling key β-lactam antimicrobial agents, particularly amongst Gram-negative bacteria.

This project gives significant insight into the antimicrobial and resistance-modifying properties of LY2183240 regioisomers and is the first study examining the activity of these agents towards prokaryotic systems. The 2,5-LY2183240 regioisomer had significant and potent activity against certain bacteria, which included multi-drug resistant staphylococci. Both regioisomers were also found to be specific inhibitors of class C β-lactamases. These findings suggest that LY2183240 provides a suitable scaffold for the design and development of compounds aimed specifically towards clinically pertinent pathogens and also as a means of tackling β-lactamase-mediated resistance.

This thesis describes research conducted in the UCL School of Pharmacy between October 2013 and September 2017 under the supervision of Dr Paul Stapleton and Professor Simon Gibbons. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are distinctly indicated. I also confirm that I have written all the text herein and have clearly indicated by suitable citation any part of this thesis that has already been published.

Signature…….Date ……

(Pedro Ernesto de Resende)

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CONFERENCES AND PUBLICATIONS PROCEDINGS

- UCL – School of Pharmacy PhD Research Day, April 24th 2015, Poster Presentation: Antimicrobial Properties of the Synthetic Legal High LY2183240.

- 12th ULLA Summer School (European University Consortium for Pharmaceutical Sciences) July 5 – 9, 2015, Paris, France. Poster Presentation: Antimicrobial Properties of a Synthetic Legal High.

- UCL – School of Pharmacy PhD Research Day, 23rd September 2016, Oral Presentation: Antimicrobial and Resistance-Modifying Properties of LY2183240 Regioisomers. Runner Up Prize of Best Presentation.

- ASM Microbe 2017, June 1 – 5, 2017, New Orleans, USA. Poster Presentation: Enzyme Kinetic Studies on LY2183240: A Specific Inhibitor of the Class C β-Lactamase from *Enterobacter cloacae*.

Manuscripts related to the data developed in this project and other research collaborations are now in progress and soon will be submitted to high-impact journals of the field.

LIST OF ABBREVIATIONS

1,5-LY2183240	5-([1,1'-biphenyl]-4-ylmethyl)-*N*,*N*-dimethyl-1*H*-tetrazole-1-carboxamide
2,5-LY2183240	5-([1,1'-biphenyl]-4-ylmethyl)-*N*,*N*-dimethyl-2*H*-tetrazole-1-carboxamide
2D	Two dimensions
3D	Three dimensions
Å	Angstrom
AMX	Amoxicillin
ATCC	American Type Culture Collection
CEF	Cefotaxime
CFU	Colony-forming units
cm	Centimetre
COSY	Correlation spectroscopy (NMR)
d	Duplet (NMR)
Da	Dalton
DEPT	Distortionless Enhancement of Polarisation Transfer (NMR)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EMCDDA	European Monitoring Centre for Drugs and Drugs Addiction
ESBL	Extended-Spectrum β-Lactamase
ESIMS	Electrospray Ionization Mass Spectrometry
FAAH	Fatty Acid Amide Hydrolase
FAS II	Fatty Acid Synthesis Type II
FPLC	Fast Protein Liquid Chromatography
g	Times Gravity or G-Force
g	Grams
HCl	Hydrogen Chloride
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HMQC	Heteronuclear single-quantum correlation spectroscopy
HPLC	High Performance Liquid Chromatography
Hz	Hertz
ISB	Iso-Sensitive Broth
J	Spin-Spin coupling constant (Hz)
KCl	Potassium Chloride
K_{cat}	Catalyst Rate Constant (turnover number)
K_i	Constant of Inhibition
K_m	Michaelis-Menten Constant
m	Multiplete (NMR)
m/z	Mass-to-charge ratio
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization - Time of Flying
MBC	Minimum Bactericidal Concentration
MeOH	Methanol
MES	2-(*N*-morpholino) ethanesulfonic acid (buffer)
MIC	Minimum Inhibitory Concentration

mL	Millilitre
mM	Milimolar
mRNA	Messenger RNA
MRSA	Methicilin Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MSSA	Methicilin Susceptible *Staphylococcus aureus*
MDR	Multi Drug Resistant
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCTC	National Collection of Type Cultures
NDM-1	New Delhi Metallo-β-Lactamase - 1
nm	Nanometre
NMR	Nuclear Magnetic Resonance
NOESY	Rotating frame nuclear Overhauser effect spectroscopy
PBP	Penicillin Binding Protein
PBS	Phosphate Buffer Saline
PDA	Photodiode Array
PDB	Protein Data Bank
PEG	Polyethylene glycol
PEI	Polyethyleneimine
pH	Potential of Hydrogen
QTOF	Quadrupole - Time of Flying
RNA	Ribonucleic Acid
s	Singlet (NMR)
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
t	Triplet (NMR)
TLC	Thin Layer Chromatography
tRNA	Transport RNA
UV	Ultraviolet
Vmax	Maximum Velocity
w/v	Weight/Volume
WHO	World Health Organization
WTA	Wall Teichoic Acid
µL	Microliter
µM	Micromolar

To my parents,
Adriana and Pedro

“*Great things are not accomplished by those who yield to trends, fads and popular opinion.*”

− Jack Kerouac

1. CHAPTER 1

Introduction

1.1 Basic Modes of Antibiotic Action

To reach the large cells numbers observed during an infection or on the surfaces of the body, microorganisms have to grow and divide, replicating repeatedly. There is immense variation across pathogen species in the number of cells required to give rise to an infection. For instance, species such as *Shigella* and *Giardia lamblia* require about 10 cells to infect a host. In contrast, species such as *Staphylococcus aureus* require 10^3 –10^8 cells in order for an infection to develop (Leggett et al., 2012). To make this possible, they must synthesize several kinds of biomolecules. Antimicrobial agents interfere with specific processes that are vital for growth and division (Neu & Gootz, 1996).

Moreover, antimicrobial agents may be either bactericidal, killing the microorganism, or bacteriostatic, inhibiting its growth. In general, bactericidal agents result in irreversible damage to a cell, in contrast, bacteriostatic agents, although they do not kill the cell, they can be extremely useful since they prevent further replication of the pathogen and allow the immune system time to mount its defences against the infection (Lee & Bishop, 2012).

Antibiotics may be categorized into 5 major modes of action: (i) interference with cell wall synthesis, (ii) inhibition of protein synthesis, (iii) interference with nucleic acid synthesis, and (iv) inhibition of a metabolic pathway and (v) membrane disruption (Tenover, 2006; Džidić et al., 2008).

1.1.1 Inhibition of Cell Wall Synthesis

The bacterial cell wall is a complex structure composed of a range of macromolecules, many of which are not found elsewhere in nature (Gale et al., 1981). Peptidoglycan is a polymer found in the cell walls of both Gram-positive and Gram-negative bacteria that is considered to be essential for survival of bacteria. β-lactam agents inhibit synthesis of the bacterial cell wall by interfering with the enzymes, penicillin-binding protein (PBPs), required for the formation of the peptidoglycan layer; this category includes penicillins, cephalosporins, carbapenems, monobactams, glycopeptides, such as vancomycin and teicoplanin that bind to the cell wall pentapeptide substrate and block transglycosylation (Walsh et al., 1993; Neu & Gootz, 1996; Tenover, 2006; Lovering et al., 2007).

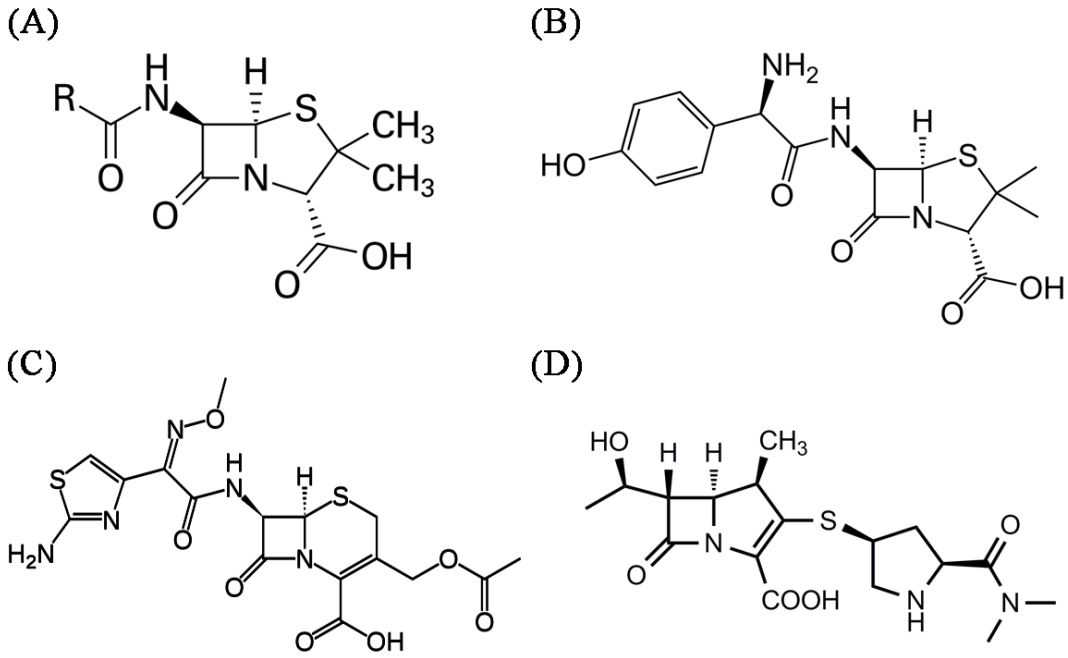


Figure 1.1. Chemical structures of β-lactam agents of different categories. (A) Penicillin core structure, "R" is the variable group; (B) Amoxicillin; (C) Cefotaxime; and (D) Meropenem.

1.1.2 Inhibition of Protein Synthesis

Many compounds have been shown to inhibit protein synthesis selectively. Definitely the preponderance of antibiotics, which act against protein synthesis do so by inhibiting functions of ribosomes – the multi-macromolecular complexes on or in which decoding of the genetic message occurs (Gale 1981). Protein synthesis is achieved by the pairing between a triplet of nucleotide bases in the incoming aminoacyl-tRNA and the complementary triplet in messenger RNA (mRNA) and subsequent joining of amino acids to form a polypeptide.

Macrolides, for instance, bind to the 50S ribosomal subunit and interfere with the polypeptide chain elongation. Aminoglycosides inhibit polypeptide chain initiation binding to the 30S ribosomal subunit. Tetracyclines bind to 30S subunit of ribosome, thereby weakening the ribosome-tRNA interaction. Chloramphenicol binds to the 50S ribosomal subunit blocking the peptidyltransferase reaction (Džidić et al., 2008). Differing from others protein synthesis inhibitors that bind to the ribosomal subunits, mupirocin possess a unique mode of action. This compound reversibly binds to the isoleucyl tRNA synthetase, especially against staphylococci and streptococci, thus

blocking protein synthesis and indirectly inhibiting RNA synthesis (Sutherland et al., 1985; Slocombe & Perry, 1991).

1.1.3 Interference with Nucleic Acid Synthesis

Antimicrobial agents can target nucleic acid (RNA or DNA) synthesis in several different ways. One of the mechanisms is at the level of nucleotide metabolism, usually by blocking *de novo* synthesis of nucleotides or by rearranging the balance of reactions concerned with interconversion of nucleotides. Other compounds may act at the level of polymerization reactions, which can directly interfere with polymerases or other enzymatic processes involved in the replication and transcription of DNA (Gale, 1980).

1.1.4 Inhibition of a Metabolic Pathway

Chemotherapeutic agents may disrupt particular metabolic pathways within the bacterial cell. Sulphonamides, for example, interrupt the folate biosynthesis pathway, which is a cofactor in nucleotide synthesis, leading to the ultimate depletion of folate and arresting cell growth (Patel et al., 2003).

1.1.4.1 Inhibition of Fatty Acid Synthesis

Within the metabolic pathway inhibition category, bacterial fatty acid synthesis represents a relevant target for potential antimicrobial agents research. The biosynthesis of fatty acids for the formation of membranes is an energy-intensive and essential aspect of cell physiology. Bacteria achieve this task employing an extremely conserved group of enzymes named the type II fatty acid synthase system (FASII) (Rock & Jackowski, 2002).

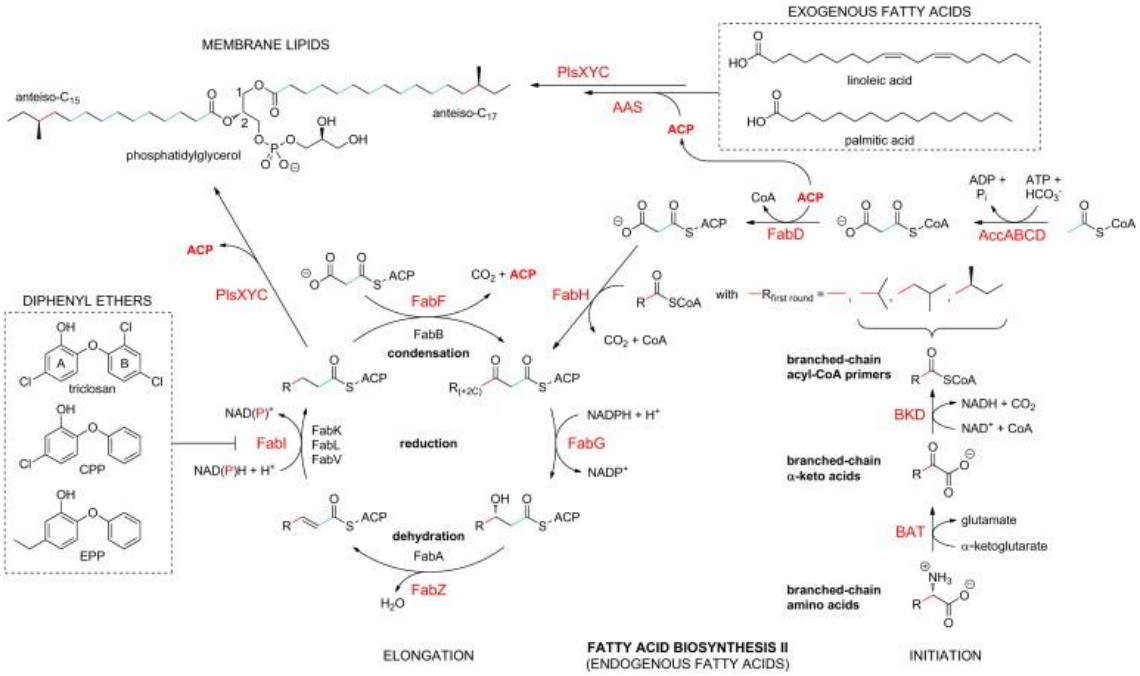


Figure 1.2. Type II fatty acid synthesis pathway in bacteria (adapted from Schiebel et al. 2012).

This system contrasts immensely with the type I FAS of eukaryotes, which is a dimer of a particular large and multifunctional polypeptide. Therefore, the bacterial pathway offers many different single sites for specific inhibition by antimicrobial agents (Heath et al., 2001).

The enzymes that regulate this pathway are generally called Fab (fatty acid biosynthesis) enzymes (Janßen & Steinbüchel, 2014). Figure 1.2 shows a simple scheme of the pathway of the bacterial FASII and some of the inhibitors previously reported (Schiebel et al., 2012). Briefly, the growth of a new acyl chain is originated by the acetyl-CoA carboxylase complex (ACC). This complex is one of the key enzymes in the fatty acid biosynthesis pathway and belongs to the family of enzymes that catalyze the intermolecular transfer of carboxyl groups via the transient formation of a carboxyphosphate intermediate covalently linked to a biotin prosthetic group (Freiberg et al., 2004). Malonyl-CoA formed is transformed to malonyl-ACP (acyl-carrier protein) where it is condensed with acyl-CoA by the enzyme FabH. The subsequent acetoacyl-ACP feeds into the elongation module where it is extended by 2 carbons with each round of the cycle by a condensation reaction with malonyl-ACP via FabF. The resulting acyl-ACP can be used by glycerol-3-phosphate acyltransferases to synthesize phospholipids (Parsons & Rock, 2011).

There are several inhibitors of FASII formerly reported in literature. Many of them are natural products that have been established as targeting ACC, as well as the

condensing enzymes FabF and FabH or the enoyl-ACP reductase FabI (D'Agnolo et al., 1973; Freiberg et al., 2006; Young et al., 2006; Zheng et al., 2007; Zheng et al., 2009; Kwon et al., 2009; Kwon et al., 2011). Moreover, some natural products have demonstrated *in vivo* efficiency in Gram-positive and Gram-negative murine infection models (Miyakawa et al., 1982; Freiberg et al., 2006; Wang et al., 2007; Wang et al., 2006).

One of the most significant FASII inhibitors is triclosan, which is a synthetic compound that has been used for decades as an antibacterial agent and has extensive use as an additive in a variety of antibacterial consumer products (Bhargava & Leonard, 1996; Rock & Jackowski, 2002). This compound specifically inhibits the enzyme FabI in the bacterial fatty acid biosynthesis pathway (Gomez Escalada et al., 2005). Moreover, it is found in formulations as diverse as toothpastes, cosmetics, antiseptic soaps, carpets, plastic kitchenware and toys (Levy et al., 1999).

Biomonitoring studies have detected triclosan in human urine, plasma, and breast milk suggesting health concerns (Adolfsson-Erici et al., 2002; Allmyr et al., 2006; Dayan, 2007; Wolff et al., 2007; Calafat et al., 2007). Nevertheless, a more recent report revealed that the exposure to triclosan in consumer products is not estimated to cause adverse health effects in children or adults who utilize these products as originally proposed (Rodricks et al., 2010).

In 2009, Brinster and coworkers published a contentious research paper claiming that bacterial fatty acid synthesis (FASII) is not a suitable antimicrobial target for Gram-positive pathogens. The authors demonstrated that in general, Gram-positive microorganisms, including streptococci, pneumococci, enterococci and staphylococci, defeated drug-induced FASII pathway inhibition when provided with external fatty acids, such as those found in human serum. This report triggered a commotion among bacterial fatty acid synthesis researchers; Balemans et al. (2010) responded presenting *in vitro* and *in vivo* data revealing that former observations are not valid for *S. aureus*, one of the most relevant Gram-positive pathogen causing numerous human infections. Moreover, the detected divergences among Gram-positive pathogens within FASII systems suggest a variety either in fatty acid biosynthesis enzymes or in the capacity for fatty acid uptake from the environment (Balemans et al., 2010).

More recently, Yao & Rock (2016) also debated about this topic presenting arguments that the situation is more complex since not all Gram-positive

microorganisms possess similar fatty acid structures as mammals. In addition, the hypothesis stated by Brinster et al. (2009) is not consistent with experimental observations that show the efficacy of FASII therapeutics against *S. aureus* using *in vivo* models (Freiberg et al., 2006; Escaich et al., 2011; Banevicius et al., 2013).

To summarize, there is still no agreement in the literature about the significance of developing FASII inhibitors as antibacterial drugs against Gram-positive pathogens. Notwithstanding, the efficiency of fatty acid synthesis inhibitors in *S. aureus* infection models should not be overlooked and there is a need to have a detailed understanding of lipid metabolism in clinically relevant pathogens (Parsons & Rock, 2011).

1.1.5 Membrane disruption

Some agents can affect the membrane permeability of the cell. Usually they have no distinction between microbial and mammalian membranes, however the fungal cell membrane demonstrated to be more flexible to selective action (Chopra, 2010). Until recently, polymyxin was the only membrane-active antibacterial agent used in human medicine, however, daptomycin, a cyclic lipopeptide, has now been introduced.

These agents act by disrupting the Gram-negative bacterial cytoplasmic membrane, potentially by attacking the exposed phosphate groups of the membrane phospholipid. This action results in a leakage of cytoplasmic contents and death of the cell (Gilleland et al., 1984).

1.2 Antimicrobial Resistance

Antimicrobial resistance is not a threat to public health but a reality. A recent report by the World Health Organization reveals that this issue is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country (WHO, 2014).

From time to time, microorganisms in general develop a natural resistance to antibiotics, and this process has been accelerated by the overuse of antimicrobials for trivial infections as well as those widely employed for animal farming and agricultural purposes (Baquero et al., 2008; Spellberg et al., 2008; Martinez, 2009).

Moreover, the pharmaceutical industry has not prioritized the development of new antimicrobial agents due to the fact that the process is expensive and any new drugs will produce limited profits when compared to other drug classes (Torjesen, 2014). Consequently, there was an antimicrobial drugs discovery gap from late 70's until beginning of 2000's (Figure 1.3), which led to a significant impact on the antibiotic resistance crisis (Lewis, 2017).

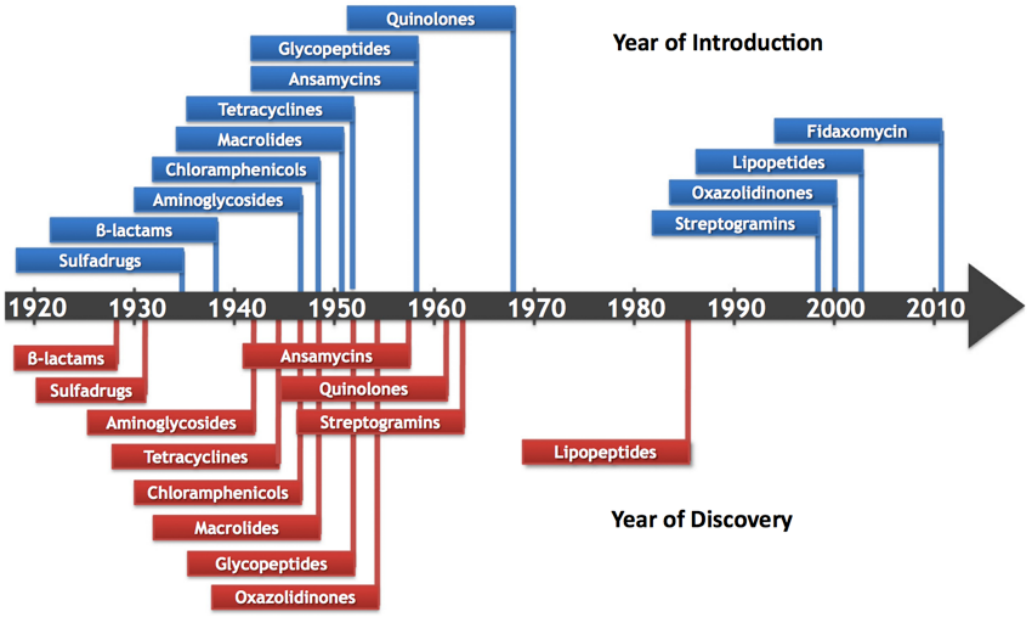


Figure 1.3. The timeline of antibiotic discovery and introduction into the clinic. This figure was adapted from Lewis (2017) and does not comprise a comprehensive listing of antibiotics, several examples have been omitted to achieve clarity. In blue, the year of introduction into clinical practice; in red, year of discovery of a major antibiotic class. The last class to be discovered, lipopeptides (daptomycin) is an exception, the compound does not hit a specific target, but rather increases membrane permeability.

To combat drug-resistant bacteria novel strategies and new targets are necessary to keep pace with ever-evolving bacterial resistance. There are recent reports mentioning different approaches to tackle this public health threat. Proteases, for instance, are essential for the proliferation and growth of bacteria and are also known to contribute to bacterial virulence. Some authors discuss that bacterial proteases, such as transpeptidases, make suitable candidates as therapeutic targets for the treatment of infectious diseases (Kaman et al., 2014; Culp & Wright, 2017). More specifically, serine hydrolases play crucial roles in prokaryotes, including pathogen

life cycle, virulence and drug resistance (Damblon et al., 1996; Silver, 2007; Steuber & Hilgenfeld, 2010; White et al., 2011; Bachovchin & Cravatt, 2012). A classic example of drug resistance mediated by serine hydrolases is β-lactamases. These enzymes are capable of inactivating β-lactam agents and they are associated with some of the most serious infectious disease issues that are currently encountered (Bush, 2013). The potential to inhibit these enzymes still remains an interesting approach and a resurgence in this topic with the focus on agents with novel mechanisms of action against a wide range of β-lactamases is currently being addressed (Drawz et al., 2014).

1.2.1 The Social Impact of Antimicrobial Resistance

Bacteria have existed on this planet for about 3.5 billion years and in that time have become adept at resisting the toxic substances that have threatened their survival (Bennett, 2008). Microbial adaptation stems from their genetic plasticity and rapid replication, making microorganisms one of the most diverse and adaptable life forms on the planet (Spellberg et al., 2008). From this framework, it seems recognizable that these organisms do not need help in creating antibiotic resistance. Nevertheless, the increase in antimicrobial resistance currently occurs due to the extensive, uncontrolled and in many cases inappropriate use of antimicrobials worldwide (Levy, 2002).

From the perspective of public health, considering the impact of microbial resistance on health and economy, it is observed that antibiotic-resistant infections double the duration of hospital stays, double mortality as well as morbidity (and presumably the costs) when compared with drug-susceptible infections (Levy & Marshall, 2004). A cost comparison of treating methicillin-resistant (MRSA) versus methicillin-susceptible (MSSA) *Staphylococcus aureus* in New York City found almost a threefold increase in mortality (21 % versus 8 %) and an economic cost increase of 22 % associated with MRSA. For all hospitalized individuals with MRSA in New York City, such costs would translate into millions of dollars (Levy, 2002).

According to the WHO (2014), microbial infections cause more than 25 % of deaths worldwide, while in developing countries the rate may reach 45 %. Furthermore, over 50 % of prescriptions are inappropriate and in many countries two thirds of antibiotics are used without a prescription. Additionally, more than 50 % of the

budget of drugs is allocated to the purchase of antimicrobials; antibiotics represent 12 % of all ambulatory requirements, which corresponds to 15 % of the 100 billion dollars spent annually on medicines (WHO, 2013; Dye, 2014). Most of these studies have been conducted within the United States of America and are based on hospital data and include the costs related to additional hospital stays and treatment, but not to early mortality. Within the USA, there are 160 million prescriptions of antibiotics, corresponding to 25 tons of antibiotics, of which about 50 % are intended for patients with the remaining being used in animals and agriculture (Wenzel & Edmond, 2000).

In the UK, after the severe increase of extended-spectrum β-lactamase (ESBL)-producing organisms such as *Escherichia coli* and *Klebsiella pneumoniae* in the 1980's and 90's, from 2001 henceforth, the situation seems to have stabilized, with similar levels of ESBLs being detected in 2006 and 2007 (Reynolds, 2009). Likewise, it is noted that there has been a marked reduction in the number of methicillin-resistant *S. aureus* (MRSA) infections in England since 2005, and a decrease in the occurrence of MRSA in comparison to the total number of *S. aureus* isolated (Reynolds, 2009). This positive result may be the outcome of the strategies implemented by the UK government in recent years, which includes improving infection prevention and control practices, optimising prescriptions, developing new drugs, treatments and diagnostics, improving professional education, training and public engagement, amongst others. (Department of Health & UK Government 2013).

Thus, it seems clear that large-scale production and use of antibiotics in clinical and veterinary medicine, poor infection control in hospitals and communities, uncontrolled use of antimicrobials in agriculture and aquaculture as well as the physician's misuse of antibiotics, are all major reasons that promote selective pressure and aggravate antibiotic resistance (Spellberg et al., 2008; Aminov, 2009; Giedraitienė et al., 2011). Whilst measures may be implemented to control antibiotic resistance development and spread, issues still remain, particularly amongst Gram-negative bacteria and the possible re-emergence of drug-resistant Gram-positive bacteria.

1.2.2 Antimicrobial Resistance Mechanisms

Since the discovery and consequently extensive use of antimicrobials, a diversity of bacterial species from different sources and origins have developed numerous mechanisms that provide microorganisms resistance to some, and in certain cases to almost all antimicrobial agents (Džidić et al., 2008).

1.2.2.1 Genetic Mechanism of Resistance

Resistance to any antimicrobial agent may be inherent or acquired by microorganisms. Inherent or intrinsic resistance is a stable genetic attribute encoded within chromosomal DNA and shared by all members of the genus, such as the AmpC β-lactamases of Gram-negative bacteria and many multi-drug resistant efflux systems (Alekshun & Levy, 2007). On the other hand, acquired resistance comprises mutations in genes targeted by the antibiotic and the transfer of resistance determinants that express a novel phenotypic feature carried on plasmids, bacteriophages, transposons, and other mobile genetic elements (McManus, 1997; Alekshun & Levy, 2007). A single mutation that gives full clinical resistance in only one step is comparatively rare, but it has been well documented for certain agents, such as quinolones, sulphonamides, streptomycin and trimethoprim (Shafran, 1990). Despite the low probability, these types of mutations persist and commonly result in an altered protein that is less susceptible to the antibiotic. For instance, this has been seen in gonococci; the ability of penicillin G to reach PBPs in the cell wall has gradually diminished, leading to low-level resistance. However, this essentiality represents an evolutionary process, and resistance by this method is relatively rare (McManus, 1997; Franklin & Snow, 2005) (McManus, 1997; Franklin & Snow, 2005). It is important to note that most of the genetic elements involved are commonly carriers of multifactorial determinants. In other words, the appearance of a new antimicrobial resistance gene must occur as a result of combined genetic processes and it is not likely to be an independent event (Dougherty & Projan, 2003). Genes coding for extended-spectrum β-lactamases (ESBLs) in Gram-negative bacteria are an interesting example of the requirement of multifactorial mutations (Gniadkowski, 2008).

Bacterial pathogens that have obtained mutations presenting antibiotic resistance frequently have a lower growth rate and are less invasive or transmissible initially than their susceptible counterparts (Wiesch et al., 2010). Nevertheless, fitness costs of resistance mutations can be enhanced by secondary site mutations. Compensatory

mutations may restore fitness in the absence and/or presence of antimicrobial agents (Brown et al., 2010; Melnyk et al., 2015).

The clear benefit to a bacterial species of acquiring new DNA is mainly because it provides a selective advantage when a new resistance attribute is acquired and expressed phenotypically. New resistance genes are transferred among bacteria and can be integrated into the bacterial chromosome and stably inherited from generation to generation, or they may be preserved on a bacterial plasmid. Plasmids may carry genes for a variety of traits, including antimicrobial resistance (Davies, 1997; McManus, 1997; Franklin & Snow, 2005; Alekshun & Levy, 2007).

New genes encoding resistance from chromosomal or plasmid DNA can be transferred among microorganisms by transduction, transformation and conjugation.

In transduction, these genes can be transported from one microbe to another through bacteriophages (bacterial viruses). Bacteriophages have a very restricted host range and are consequently much less successful in spreading resistance within a bacterial population (Goodman et al., 2011; McManus, 1997).

Bacteria can acquire DNA from their environment in a process called transformation. Like transduction, the compatibility range between donor and recipient is limited. Transformation is the least important mechanism for antibiotic resistance development, nonetheless it is the molecular basis of penicillin resistance in some species like pneumococci (Stratton, 1997). It is believed that they have been introduced from viridans group streptococci and integrated into the resident penicillin binding protein genes by homologous recombination, although it has proven difficult to identify the source or sources of the divergent region (Spratt, 1994). Lastly, conjugation is by far the most important process that implicates the dissemination of antimicrobial resistance including the majority of reports of bacterial gene transfer in the environment (Llosa & de la Cruz, 2005).

1.2.2.2 Biochemical Mechanisms of Resistance

Bacterial resistance genes are expressed phenotypically as biochemical interference with an antimicrobial's mode of action, ensuing decreased activity against the strain with the resistant trait. There are several types of resistance mechanisms that bacteria have acquired over the years in order to survive (McManus, 1997). The prevalence of these mechanisms depends on the nature of the antibiotic, its target site, the

bacterial species and whether it is mediated by a resistance plasmid or by a chromosomal mutation (Džidić et al., 2008).

Some of the mechanisms are the direct inactivation of the active antibiotic molecule, including the production of enzymes that degrade or modify the drug itself. Biochemical strategies are hydrolysis, group transfer, and redox mechanisms (Wright, 2005; Džidić et al., 2008). Several enzymes are known to destroy antibiotic activity by targeting and cleaving hydrolytically susceptible chemical bonds. A typical example is the β-lactamases, which cleave the β-lactam ring of the penicillin and cephalosporin antibiotics. A wide range of Gram-negative and Gram-positive bacteria produce such enzymes, and more than 200 different β-lactamases have been identified (Richmond & Sykes, 1973; Bush & Jacoby, 2010). This mechanism of resistance is addressed in more detail below (See Section 1.1.2.3).

Alterations in the target sites of antibiotics are also a common mechanism of resistance whereby the antimicrobial agents are no longer able to bind to their target effectively. Target site changes frequently arise as a consequence of spontaneous mutation of an encoding gene and selection in the presence of an antibiotic. Mutations in RNA polymerase and DNA gyrase genes are examples of resistance to rifamycins and quinolones, respectively. In some cases, genetic exchange such as conjugation, transduction and transformation may be involved in the acquisition of resistance. The *mecA* gene, encoding PBP2a enzyme, conferring methicillin resistance in *Staphylococcus aureus* was acquired through genetic acquisition from an unconfirmed organism (Lambert, 2005). Another example is the mechanism of resistance of sulphonamides. These antimicrobial agents, including trimethoprim, inhibit the enzyme dihydrofolate reductase (DHFR) by competitively binding to its catalytic site (Van Hoek et al., 2011). High-level resistance is commonly accomplished by a bypass mechanism through the action of an acquired gene encoding an alternative enzyme, which is a non-allelic and drug-insusceptible variant of a chromosomal DHFR. These plasmid-mediated DHFRs emerged in Gram-negative bacteria within several years of the clinical introduction of the drug (Huovinen et al., 1995; Sköld, 2000; Sköld, 2001).

Another category of resistance mechanisms is associated with transport of the agent into the cell. Reduced outer membrane permeability results in reduced antibiotic up-take. Generally, this type of resistance is mediated by modifications in the lipid or protein composition of the outer membrane of the bacterial cell (Delcour, 2009).

Moreover, efflux pumps, for instance, which are membrane proteins, export antibiotics out of the cell and keep their intra-cellular concentrations at low levels. The reduced uptake and active efflux give rise to low-level resistance in many clinically important bacteria (Kumar & Schweizer, 2005; Džidić et al., 2008).

1.2.2.3 β-Lactamases

The development of bacterial enzymes with the ability to hydrolyse β-lactams remains one of the most substantial threats to the effectiveness of this essential class of antimicrobial agents (Bush et al., 2011; Drawz et al., 2014). In 1940, even before the clinical use of penicillin, the first β-lactamase was identified in *Escherichia coli* and it was defined as an enzyme capable of destroying penicillin (Abraham & Chain, 1940). Notwithstanding, this report was not recognized as a significant threat to drug development since penicillin was utilized only for Gram-positive bacterial-related infections. Four years later, a β-lactamase was reported from *S. aureus*, and subsequently became a significant clinical problem until the development of β-lactamase stable anti-staphylococcal penicillin-derivatives (Kirby, 1944). More recently, the number of distinctive protein sequences for these enzymes, amongst a wide range of organisms, surpassed 890, and more than 1300 diverse β-lactamases have now been identified in clinical isolates (Bush et al., 2011; Bush, 2013; Farina et al., 2014).

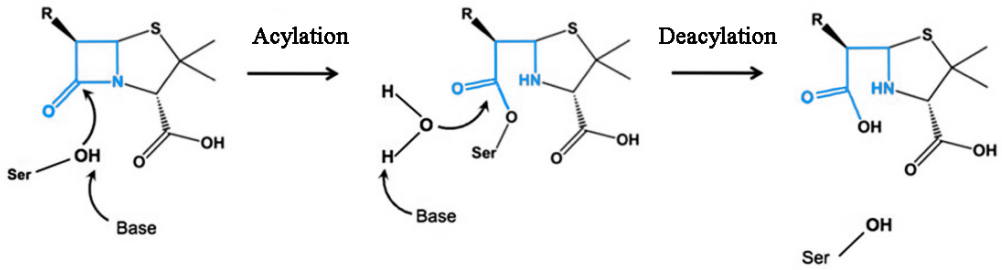


Figure 1.4. General scheme of the catalytic mechanism of serine β-lactamases adapted from Stojanoski et al. (2016). The β-lactam ring is shown in blue. The serine nucleophile is activated via a general base and attacks the carbonyl of the β-lactam ring in the acylation step. This results in formation of the covalent acyl-enzyme complex. In the deacylation step the complex is resolved by a catalytic water molecule, which is activated by the general base.

1.2.2.3.1 Classification

The classification of β-lactamases is a constant research topic and two classification schemes are currently in use.

The molecular classification, also known as the Ambler classification, is based on the amino acid sequence and divides β-lactamases into classes A, B, C, and D enzymes. Classes A, C and D utilize a serine residue in the catalytic site for β-lactam ring hydrolysis (Figure 1.4). Conversely, class B enzymes, also known as metallo-β-lactamases, require divalent zinc ions for substrate hydrolysis (Ambler, 1980; Bush & Jacoby, 2010). Notably, serine β-lactamases belong to the serine hydrolases super family and represent the largest group of β-lactamases of relevant research interest.

The second classification system is based on functional characteristics and it was presented by Bush, Jacoby and Medeiros (1995) as a refinement of earlier schemes. There are three main clusters of enzymes that are defined by their substrate and inhibitor profiles. For example, Group 1 are cephalosporinases that are not well inhibited by clavulanic acid; Group 2 penicillinases, cephalosporinases, and broad spectrum β-lactamases that are normally inhibited by active site-directed β-lactamase inhibitors; and finally, Group 3 are metallo-β-lactamases that hydrolyse penicillins, cephalosporins, and carbapenems and that are weakly inhibited by almost all β-lactam-containing molecules. Within each group there are sub-groups denoting an extension of hydrolytic activity, e.g., Group 2be, with the letter "e" for extended spectrum of activity, represents the ESBLs, which are able of inactivating third-generation cephalosporins (Bush et al., 1995).

The molecular classification is more frequently used among β-lactamase researchers, and consequently adopted in this study.

1.2.2.3.1.1 Class A

Normally, class A enzymes are susceptible to the commercially available β-lactamase inhibitors such as clavulanic acid and tazobactam. Nonetheless the carbapenemase KPC from *K. pneumoniae* is a significant exemption to this generality (Papp-Wallace et al., 2010). In 1965, the first plasmid-mediated β-lactamase in *E. coli* was reported and denoted “TEM” derived from the first three letters of the patient’s name from whom the organism was isolated (Datta & Kontomichalou, 1965). Another β-lactamase named, SHV, from the term sulfhydryl

reagent variable, is commonly found in *K. pneumoniae* (Drawz et al., 2010). The name's origin indicates that *p*-chloromercuribenzoate (which binds sulfhydryl groups) inhibits hydrolysis of cephaloridine but not of benzylpenicillin (Matthew, 1979). However, currently, many of the three letter β-lactamases names have lost their historical meanings (Livermore, 1995). These two β-lactamases are usually identified in clinical isolates of *E. coli* and *K. pneumoniae*, pathogens responsible for urinary tract, hospital-acquired respiratory tract, and bloodstream infections (Thomson & Amyes, 1993; Gorbach, 1994; Buynak, 2006). Although SHV-1 and TEM-1 share almost 70% sequence homology, the catalytic site of SHV-1 is about 0.7 to 1.2 Å broader than that of TEM-1, which may have essential structural implications, especially related to the substrate profiles of SHV variants (Tzouvelekis & Bonomo, 1999).

It is noteworthy that these enzymes may exist as extended-spectrum β-lactamase (ESBL) variants.

ESBLs are a family of enzymes capable of hydrolyzing penicillins, cephalosporins, and monobactam antibiotics, whereas the host bacteria would still be sensitive to cephamycins, carbapenem antibiotics, and inhibitors of the enzymes (Han et al., 2016). The most common ESBL genotypes include the class A, TEM, SHV and CTX-M, as well as the class D, OXA enzymes (Paterson & Bonomo, 2005; Perez et al., 2007; Liakopoulos et al., 2016).

A typical hydrolytic reaction mechanism of a class A β-lactamase involves several steps. Briefly, after formation of the Henri-Michaelis complex, the active-site serine executes a nucleophilic attack on the carbonyl of the β-lactam agent, which results in a high-energy tetrahedral acylation intermediate. This intermediate transitions into a lower-energy covalent acyl-enzyme following protonation of the β-lactam nitrogen and cleavage of the C-N bond. After that, an activated water molecule attacks the covalent complex and leads to a high-energy tetrahedral deacylation intermediate, and consequently the hydrolysis of the bond between the β-lactam carbonyl and the oxygen of the nucleophilic serine regenerates the active enzyme and releases the inactive β-lactam. The acylation and deacylation processes (Figure 1.4) require the activation of the nucleophilic serine and hydrolytic water, respectively (Strynadka et al., 1992; Damblon et al., 1996; Drawz et al., 2010).

1.2.2.3.1.2 Class B (Metallo-β-Lactamases)

Metallo-β-lactamases are metallo-enzymes that require one or two zinc ions within the active site for their activity. In general, these enzymes use the hydroxyl group from a water molecule that is coordinated by Zn^{2+} to hydrolyze the amide bond of a β-lactam. They belong to the molecular classification of class B β-lactamases according to their amino acid sequence homology, and to Group 3 according to the Bush-Jacoby-Medeiros classification scheme, which is based on their substrate profiles (imipenem hydrolysis), universal inhibition by ethylene diamine tetra-acetic acid (EDTA) and absence of inhibition by serine β-lactamase-susceptible inhibitors (Ambler, 1980; Bush et al., 1995; Rasmussen & Bush, 1997; Maltezou, 2009).

These types of β-lactamases can hydrolyze all classes of β-lactams agents, excluding monobactams, and they are unique for their continuous and effective carbapenem-hydrolyzing activities. This represents a distressing feature not only because carbapenems still are stable against the majority of serine β-lactamases, but also due to the fact they are the antimicrobial agents with the widest range of activities (Bebrone, 2007).

The first report of this type of enzyme was in a *Bacillus cereus* species in 1966 when it was demonstrated that EDTA inhibited the cephalosporinase activity produced by this organism (Sabath & Abraham, 1966). Very few studies involving more selective metallo-β-lactamases inhibitors were performed during the next two decades, possibly because this class of enzymes was not considered to be a dangerous clinical threat (Mojica et al., 2016). Nevertheless, this situation dramatically changed in the late 1980's, with the emergence of metallo-β-lactamases in clinically relevant pathogens, including *Bacteroides fragilis*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Serratia marcescens*, and *Elizabethkingia meningoseptica* (Rasmussen et al. 1990; Watanabe et al. 1991; Massidda et al. 1991; Osano et al. 1994). Furthermore, a silent gene coding for a metallo-enzyme was discovered in *Bacillus anthracis* as well as *Klebsiella pneumoniae* clinical isolates from Japan (Senda et al. 1996; Rossolini et al. 1998; Cornaglia et al. 1999).

The rise and propagation of acquired metallo-β-lactamases among general Gram-negative pathogens such as members of the family *Enterobacteriaceae, P. aeruginosa*, and *Acinetobacter* species turned the circumstances more grave and intricate (Walsh et al., 2005; Bebrone, 2007). Additionally, many of the metallo-

enzyme genes are present in environmental species, which consequently comprise reservoirs of β-lactam resistance genes (Rossolini et al., 2001; Simm et al., 2001; Stoczko et al., 2006).

The metallo-β-lactamases are divided into three subclasses (B1, B2, B3) based on primary amino acid sequence homologies (Galleni et al., 2001; Garau et al., 2004). Subclass B1 encompasses a more prevalent number of identified metallo-enzymes and embraces the well-studied *Bacillus cereus* BcII enzyme and the clinically relevant and transferable IMP-, VIM-, and NDM-type enzymes (Palzkill, 2013).

More specifically, the increasing occurrence of carbapenem-resistant *K. pneumoniae* is of primary concern and have been reported globally (Lee et al., 2016). The New Delhi Metallo-β-lactamase, NDM-1, was first identified in a Swedish patient returning from India (Yong et al., 2009). Since then, it has been shown to be present at increased frequency within *Enterobacteriaceae* in India and has successively been revealed to be present in clinical isolates in a number of countries worldwide (Palzkill, 2013). The $bla_{\text{NDM-1}}$ gene has been located on numerous plasmid types and it can be transferred among Gram-negative bacteria by conjugation (Göttig et al., 2010).

To summarize, metallo-β-lactamases are now considered a therapeutic challenge and the understanding of their alarming attributes is essential in the fight to overcome them.

1.2.2.3.1.3 Class C

AmpC β-lactamases are clinically significant cephalosporinases encoded on the chromosome of many *Enterobacteriaceae* species, including *E. coli*, *C. freundii* and *E. cloacae* and a few other organisms. They can give rise to resistance to several β-lactams such as cephalothin, cefazolin, cefoxitin, most penicillins, and β-lactamase inhibitor/β-lactam combinations (Jacoby, 2009). In many bacteria species, AmpC enzymes are inducible and can be expressed at high levels by mutation. Overexpression gives resistance to broad-spectrum cephalosporins becoming a serious problem particularly in infections with *E. aerogenes* and *E. cloacae*, where an isolate originally susceptible to these antimicrobial agents may develop resistance upon therapy (Chow & Shlaes, 1991; Lee et al., 1991; de Champs et al., 1993; Tzouvelekis et al., 1994; Yigit et al., 2002; Thiolas et al., 2005; Jacoby, 2009).

In several organisms, including *C. freundii* and *E. cloacae*, but not *E. coli*, β-lactamase expression may increase in the presence of certain β-lactam antibiotics, in a process referred to as induction. β-Lactamase induction by β-lactams, such as cefoxitin, imipenem and meropenem, occurs by the drugs crossing the outer membrane through porins, entering the periplasmic space, and interacting with target PBPs that form stable covalent complexes leading to inactivation (Beadle et al., 2001). An increase in pools of 1,6-anhydromuropeptides is then observed, and AmpD is unable to efficiently process the higher levels of cell wall fragments (Figure 1.5). The anhydro-MurNAc-peptides replace UDP-MurNAc-pentapeptides bound to AmpR, causing a conformational change in the protein. AmpR is converted into a transcriptional activator, AmpC is expressed at higher levels, and AmpC accumulates in the periplasmic space. When the amount of β-lactam decreases below "inducing levels," the cytoplasmic pool of anhydroMurNAc-peptides also decreases, and AmpD is able to efficiently cleave these peptides, restoring wild-type AmpC expression (Figure 1.5).

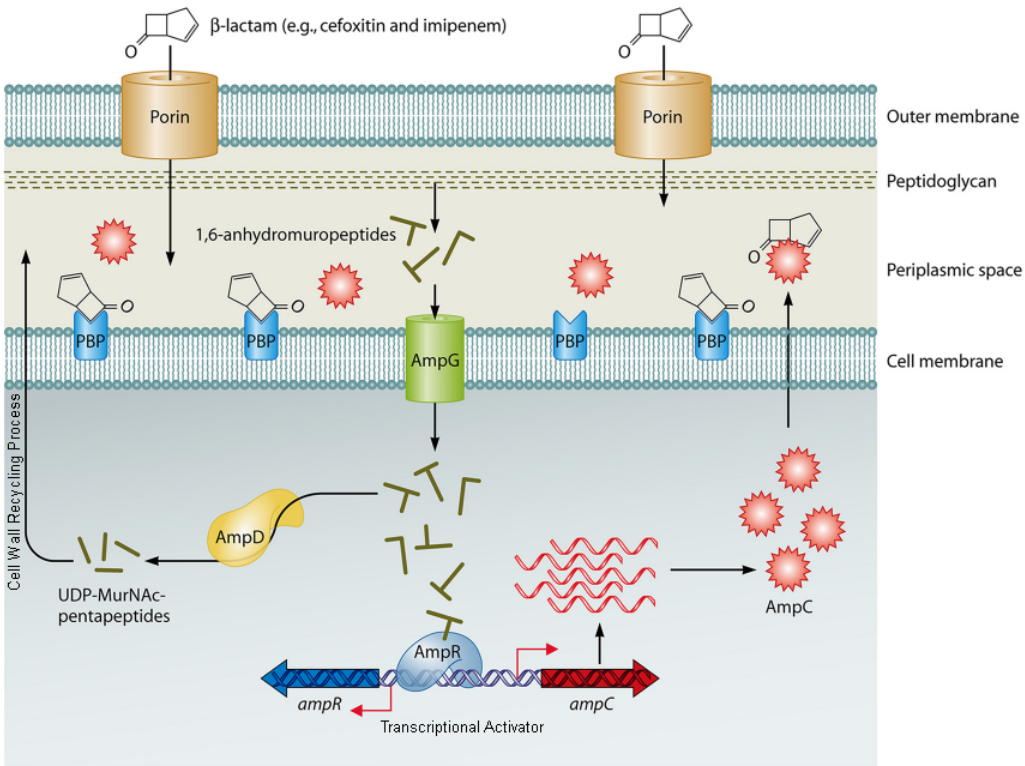


Figure 1.5. Mechanisms involved in regulation of AmpC expression. This figure is adapted from Lister et al. (2009) and portrays the present information acquired from studies with members of the *Enterobacteriaceae* family.

The fundamental evidence regarding the class C hydrolytic mechanism indicates that the residue Tyr^{150} acts as a general base by increasing the nucleophilicity of Ser^{64} for acylation (Oefner et al., 1990). Previous studies of the deacylation mechanism indicated that Tyr^{150} also acts as the catalytic base, accepting a proton from the deacylating water (Dubus et al., 1994; Dubus et al., 1996). In a study conducted using a molecular simulation method, more specifically employing mixed quantum mechanics/molecular mechanics calculations suggested that Tyr^{150} interacts with Lys^{67} in a conjugate base fashion (Gherman et al., 2004). Nevertheless, the crystal structure of the *E. coli* AmpC in complex with a deacylation transition state analog revealed that Tyr^{150} remains protonated throughout the reaction and therefore is unlikely to be the anionic base.

Figure 1.6 depicts the active site of an AmpC β-lactamase showing the main catalytic residues involved in the β-lactam ring hydrolysis, such as Ser^{64}, Tyr^{150}, Thr^{316} and Lys^{315}.

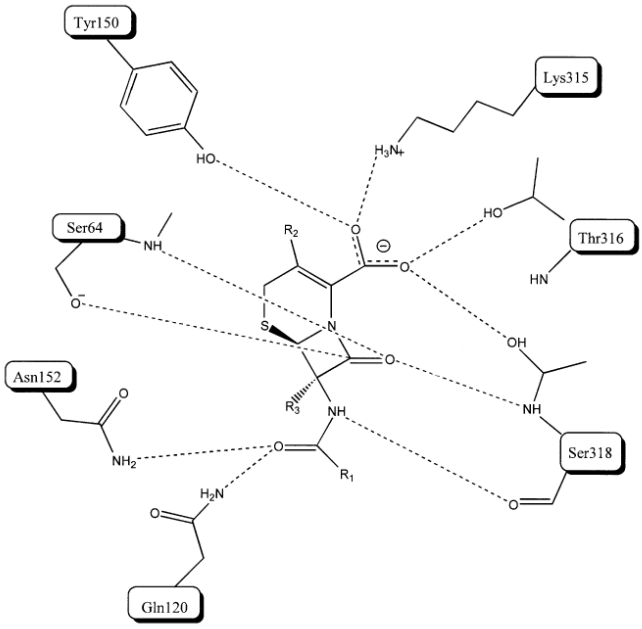


Figure 1.6. Hydrogen bond interactions into the catalytic site of *E. cloacae* P99 adapted from Fenollar-Ferrer et al. (2002). All the interactions are valid for β-lactams and derivatives.

1.2.2.3.1.4 Class D

Among the classes of β-lactamases, class D is the least studied so far despite its significance in the clinic (Maveyraud et al., 2000). This class was firstly classified as oxacillinases due to their capability to hydrolyze oxacillin at a rate of at least 50 % of

that of penicillin G, as compared to the slow hydrolysis of oxacillin by classes A and C β-lactamases (Danel et al., 2007).

The most representative feature of this β-lactamase class appears to be the presence of a conserved carboxylated lysine residue in the active site (Golemi et al., 2001; Fisher et al., 2005). Moreover, some OXA enzymes have been established to form dimers, demonstrating a biphasic kinetic behavior, e.g., it is dependent on the enzyme existing as a highly active dimer at high concentration and as a less active monomer at low concentration (Danel et al., 2001; Vercheval et al., 2010).

1.2.2.4 β-Lactamases Inhibitors

Two main approaches have been accepted to overcome the fast increase in β-lactamase-mediated resistance. The first strategy is the discovery or design of new β-lactam antibiotics able to avoid enzymatic inactivation by β-lactamases. The second strategy is the finding and design of β-lactamase inhibitors that can be used in combination with traditional β-lactam antibiotics, allowing the β-lactam antibiotics to inhibit penicillin-binding proteins (PBPs) (Farina et al., 2014). The latter strategy is effective using agents designed to bind especially at the active site of the enzyme. This approach can take two forms: (1) design substrates that reversibly and/or irreversibly bind to the enzyme with high affinity but form unfavourable steric interactions as an acyl-enzyme; or (2) create mechanism-based or irreversible, also called "suicide inhibitors" (Drawz et al., 2010).

Reversible inhibitors can be divided into three main types: competitive, non-competitive and uncompetitive (Segel, 1975; Cornish-Bowden et al., 2002).

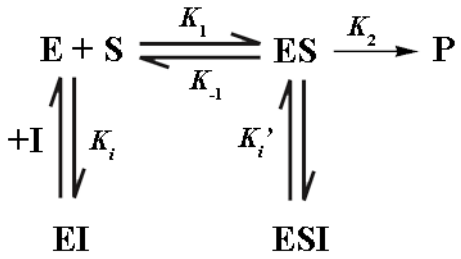
A number of graphic methods have been described for determining the mode of inhibition of a particular molecule. Of these, the double reciprocal or Lineweaver-Burk plot is the most straightforward means of diagnosing inhibitor modality (See Chapter 2, section 2.3.13.3) (Copeland, 2000). Figure 1.7 illustrates these types of inhibition as well as the graphic representations of each effect within Lineweaver-Burk plots. Competitive inhibition denotes the case of the inhibitor binding particularly to the free enzyme and not at all to the enzyme-substrate complex. In other words, the inhibitor and substrate compete for the same enzyme form and normally bind in a mutually exclusive manner; which means the free enzyme binds either a molecule of inhibitor or a molecule of substrate, but not both concomitantly

(Copeland, 2000). This type of inhibition can be overcome by sufficiently high concentrations of substrate (V_{max} remains constant), for instance, by surpassing the inhibitor. Nevertheless, the Michaelis-Menten constant (K_m) will increase as it takes a higher concentration of the substrate to reach the K_m, or half the V_{max}.

In non-competitive inhibition, the inhibitor displays binding affinity for both the free enzyme and the enzyme-substrate complex. As a result, the extent of inhibition depends only on the concentration of the inhibitor. V_{max} (maximum velocity) will decrease due to the inability for the reaction to proceed as efficiently, but K_m will remain the same, as the actual binding of the substrate, by definition, will still function properly.

Lastly, uncompetitive inhibitors bind solely to the enzyme-substrate complex, rather than to the free enzyme form (Figure 1.7). This mode of inhibition decreases V_{max} as a result of removing activated complex and K_m decreases due to better binding efficiency and the effective elimination of the ES complex hence decreasing the *Km,* which indicates a higher binding affinity.

For reversible inhibition, the reaction can be described as indicated below in Equation 1, where an enzyme (E) binds to its substrate (S) to form the enzyme-substrate binary complex ES. During catalysis, this complex breaks down to release product (P) and free the enzyme. The inhibitor (I) can bind to either E or ES with the dissociation constants K_i or K_i', respectively (Segel, 1975).



(1)

As mentioned before, irreversible "suicide inhibitors" can permanently inactivate the β-lactamase via secondary chemical reactions in the enzyme catalytic site. Equation 2 represents a general mechanism of irreversible inhibitors (I) leading to permanent enzyme inactivation.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E:I \xrightarrow{k_2} E - I \xrightarrow{k_3} E - I^* \qquad (2)$$

The rate constants for each step are represented by k_1, k_{-1}, k_2, and k_3. More specifically, k_1 and k_{-1} are the association and dissociation rate constants for the pre-acylation complex, respectively; k_2 is the acylation rate constant, and k_3 is the deacylation rate constant (Drawz et al., 2010).

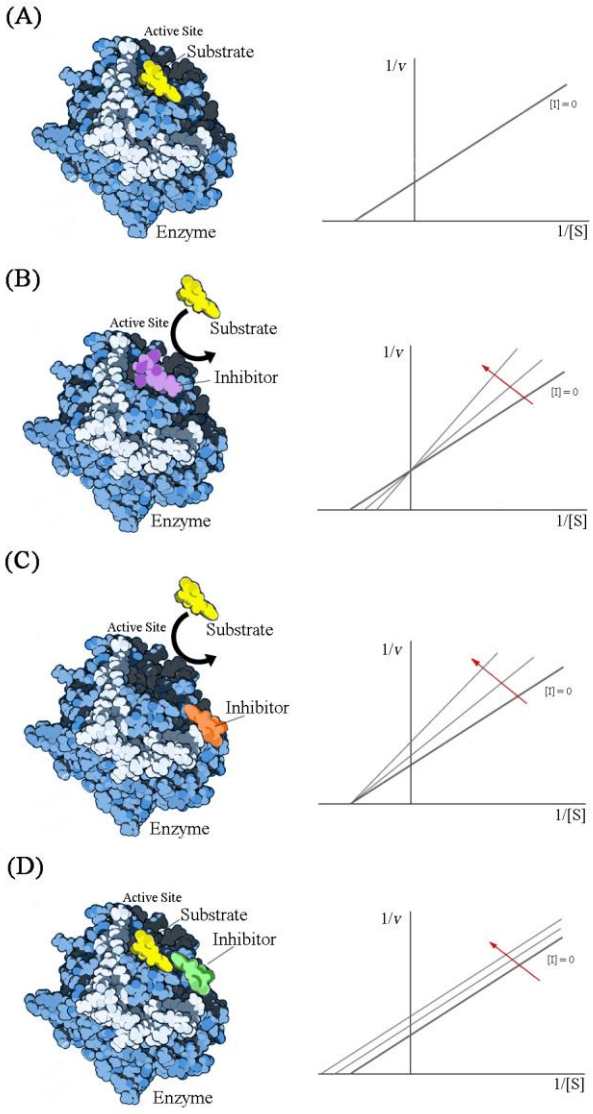


Figure 1.7. Graphic representations of basic enzyme-substrate interactions. (A) Normal binding, (B) competitive inhibition, (C) non-competitive inhibition, and (D) uncompetitive inhibition. The red arrow denotes the increase of the inhibitor concentration.

The first β-lactamase inhibitor introduced into clinical medicine was clavulanic acid (Figure 1.8 A), being isolated from the microorganism *Streptomyces clavuligerus* in the 1970s, almost 50 years ago (Reading et al., 1977). This inhibitor revealed no significant antimicrobial activity alone, but when combined with amoxicillin it lowered considerably the amoxicillin MICs against a wide range of pathogens including *S. aureus*, *K. pneumoniae*, *Proteus mirabilis*, and *E. coli* (Brown, 1986).

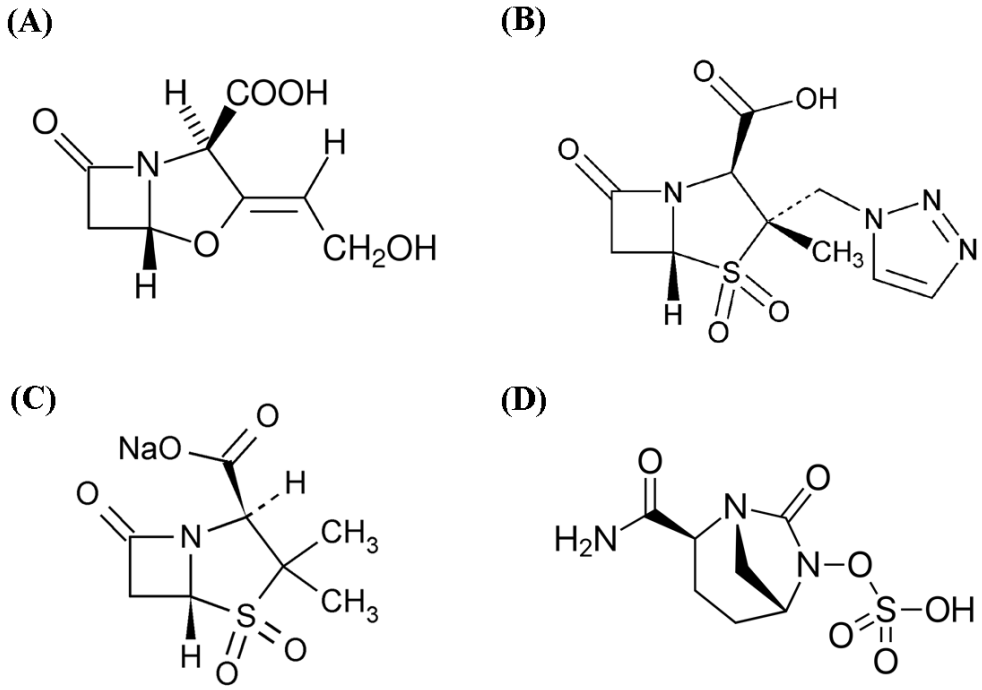


Figure 1.8. Chemical structures of the main β-lactamases inhibitors currently used. (A) Clavulanic acid; (B) Tazobactam; (C) Sulbactam and (D) Avibactam.

Sulbactam and tazobactam (Figure 1.8B and C) are penicillinate sulfones that were subsequent developed as synthetic compounds in the late 1970's and beginning of 1980's (English et al., 1978; Fisher et al., 1980). All three β-lactamase inhibitors have a β-lactam ring and share structural similarity with the antimicrobial agent penicillin (See Figure 1.1A). They are effective against many susceptible organisms expressing class A β-lactamases, including CTX-M and the ESBL derivatives of TEM-1, TEM-2, and SHV-1, but they are usually less efficient against class B, C, and D β-lactamases (Bush, 1988; Philippon et al., 1989; Paterson & Bonomo, 2005; Buynak, 2006; Cantón & Coque, 2006; Drawz et al., 2010).

Avibactam is a novel non-β-lactam inhibitor with higher potency when compared to clavulanic acid, sulbactam, and tazobactam. This inhibitor has remarkable low IC_{50} values and decreased reactivation rates for the clinically relevant class A and C β-lactamases including TEM-1, KPC-2, and P99 and the AmpC from *P. aeruginosa* as well as the ESBL CTX-M-15 (Ehmann et al., 2012; Ehmann et al., 2013; Lahiri et al., 2013; Drawz et al., 2014; Choi et al., 2016).

Table 1 depicts the kinetic properties of some relevant β-lactamases in the presence these inhibitors. This table was adapted from Drawz et al. (2010), with avibactam activity included.

Currently, there are clinically available β-lactam/β-lactamase inhibitor combinations including amoxicillin-clavulanate, ticarcillin-clavulanate, ampicillin-sulbactam, cefoperazone-sulbactam, piperacillin-tazobactam and more recently ceftazidime-avibactam (Fass & Prior, 1989; Corbella et al., 1998; Chetchotisakd et al., 2001; Geddes et al., 2007; Zhanel et al., 2013).

More specifically, the inhibitors clavulanic acid, tazobactam, and sulbactam have found widespread clinical use; still, poor activity has been demonstrated against class D ESBLs and AmpC enzymes, with exception of avibactam combinations (Bassetti et al., 2008; Levasseur et al., 2012). The combination of ceftazidime-avibactam is a novel β-lactam-β-lactamase inhibitor with activity against carbapenem-resistant *Enterobacteriaceae* that produce carbapenemases (Zhanel et al., 2013; Shields et al., 2017). Nonetheless, there are already reports of cases of ceftazidime-avibactam resistance developed during treatment of carbapenem-resistant *Enterobacteriaceae* infections (Humphries et al., 2015; Livermore et al., 2015; Haidar et al., 2017).

In this context, it is necessary continually to develop new strategies and compounds to tackle the spread of β-lactam-resistant pathogens and the increasing number of infections that are not clinically treatable.

Table 1. Kinetic properties of clinically relevant β-lactamases in the presence of inhibitors, adapted from Drawz et al. (2010).

β-lactamase	Class	Clavulanate		Sulbactam		Tazobactam		Avibactam	
		K_i (μM)	IC_{50} (nM)	K_i (μM)	IC_{50} (nM)	K_i (μM)	IC_{50} (nM)	K_i (μM)	IC_{50} (nM)
TEM-1	A	0.1	60	1.6	900	0.01	97		8[c]
SHV-1	A	1	12	8.6	12,000	0.07	150		
SHV-4	A								3[d]
SHV-5	A		20		1,800		80		
PC1	A		30		80		27		
CTX-M-2	A		200		2,100		20		
CcrA	B		> 500,000		> 500,000		400,000		
P99	C		> 500,000		5,600		8.5	7.0[a]	80[c]
CMY-2	C	4,365		101		50		26[b]	
OXA-1	D		1,800		4,700	380	1400		
OXA-2	D		1,400	0.1	140		10		

[a] Ehmann et al. (2013)

[b] Papp-Wallace et al. (2014)

[c] Bonnefoy et al. (2004).

[d] Stachyra et al. (2010)

1.3 Natural Products: 'Legal Highs'

In general, phytochemicals are a rational natural product alternative for investigation as potential antibacterial and resistance-modifying agents. Plants are known to produce abundant antibacterial metabolites as part of their protective mechanism against microorganisms in their environment (Ahuja et al., 2012). Moreover, antimicrobial agents from plants have a large prospect for biotechnological applications and have enlarged significantly in recent years with new information on their distribution, synthesis, regulation, *in vivo* function, and mechanism of action. With more than a quarter of a million species and a particularly diverse specialized metabolism, the richness of plant antimicrobials has barely been explored (Sampedro & Valdivia, 2014).

'Legal highs' are natural products from plant or fungal sources as well as synthetic compounds (tablets or powders) used as recreational drugs (Arunotayanun & Gibbons, 2012). These products are extensively available online resulting in the rise of the sales and enlarging consumption of these materials worldwide (EMCDDA, 2011). Nonetheless, a survey made in several databases revealed little information about the phytochemical composition, biological activities, and toxicology of these products. Most of the work that has been described has focused on the influence of these agents on the central nervous system. Their applications in other areas such as antimicrobial and resistance-modifying activities have yet to be explored in detail. Previous studies have shown that plants with psychoactive properties have proven to possess antimicrobial properties. *Datura metel*, a member of the *Solanaceae* family, contains tropane alkaloids, which are exploited traditionally for their hallucinogenic characteristics. Studies have found compounds derived from this species possess antibacterial action against a wide range of microbes. For instance, aqueous and ethanol leaf extracts are shown to have an MIC of 20 µg/mL against *S. aureus*, *P. aeruginosa* and *E. coli* (Akharaiyi, 2011).

Trichocereus peruvianus and *Trichocereus pachanoi* popularly known as Peruvian Torch cacti and San Pedro, respectively, are South American species belonging to the *Cactaceae* family (Figure 1.9). They are well-known psychoactive mescaline-containing cacti and commonly used in shamanistic treatments by decoction of sliced pieces of the plants. The few reports found in the literature indicate that mescaline is a major component and key constituent for their psychoactive activities (Halpern,

2044; Arunotayanun & Gibbons, 2012).

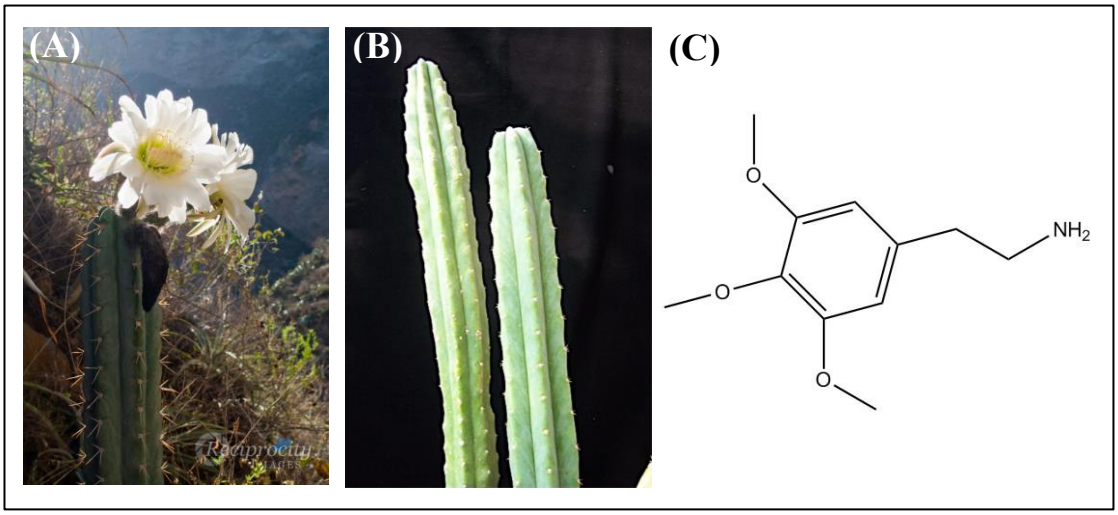


Figure 1.9. Photograph by Jason Langley (Font: Reciprocity Images®) of **(A)** *Trichocereus peruvianus*, also known as Peruvian Torch cactus. **(B)** *Trichocereus pachanoi* photographed by Cal Lemke (Font: University of Oklahoma, Department of Microbiology and Plant Biology), **(C)** mescaline, the major component present in these species and responsible for psychoactive activities.

Kratom is the popular name of the species *Mitragyna speciosa* (Rubiaceae). Mitragynine is the major alkaloid in this psychoactive plant grown in Southeast Asia, present in concentrations up to 66% in crude extracts and possesing analgesic properties (Figure 1.10) (Takayama, 2004; Arunotayanun & Gibbons, 2012).

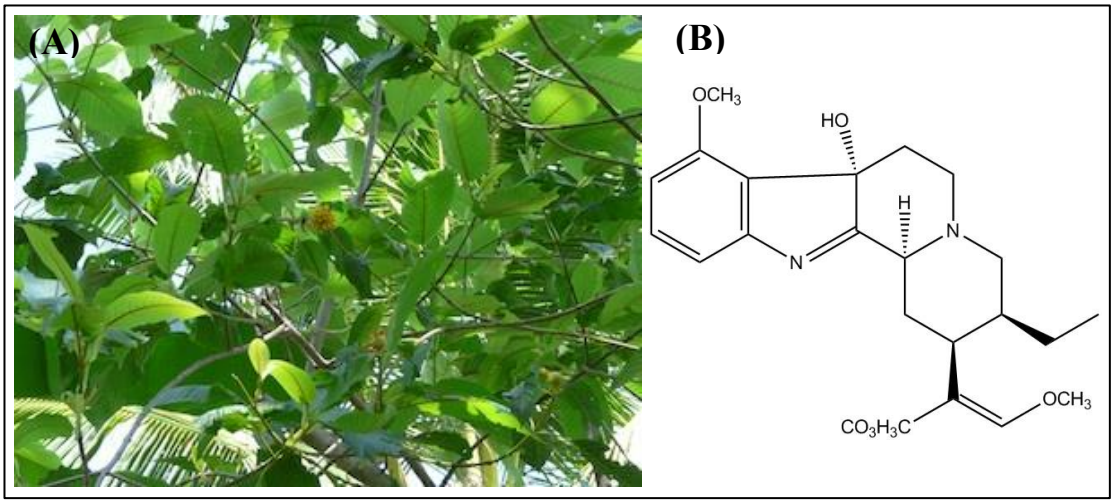


Figure 1.10. (A) Photograph of kratom (*Mitragyna speciosa*) by Dr István Ujváry, and mitragynine **(B)**, the main compound.

Argyneia nervosa also known as Hawaiian Baby Woodrose is a species used in

Ayurvedic medicine (Figure 1.11), and some reports indicate that the seed extracts show hypoglycemic, anti-fungal (Mishra, 1978) and antibacterial activity against both Gram-positive and Gram-negative organisms (Akhtar, 1992; Arunotayanun & Gibbons, 2012).

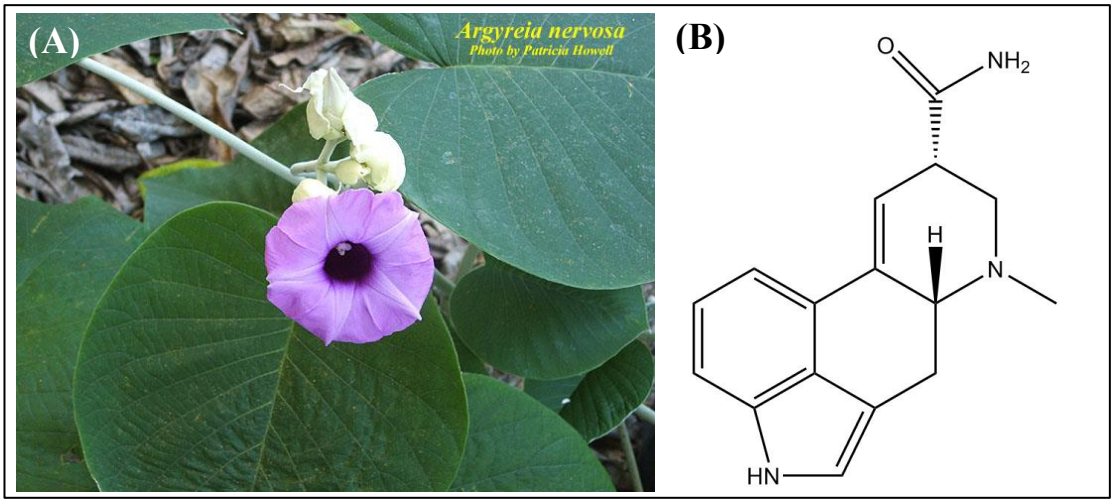


Figure 1.11. (A) *Argyneia nervosa* photographed by Patricia Howell (Font: Atlas of Florida Vascular Plants). **(B)** Lysergic Acid Amide (LSA), precursor of Lysergic Acid Diethylamide (LSD), a potent hallucinogenic compound.

Banisteriopsis caapi is one of the main ingredients of the sacred drink Ayahuasca (Figure 1.12). It is an Amazon native species and belongs to the *Malpighiaceae* family. The major components reported in this plant are β-carboline alkaloid derivatives, which mainly include harmine, harmaline, and tetrahydroharmine (McKenna, 2004, Arunotayanun & Gibbons, 2012).

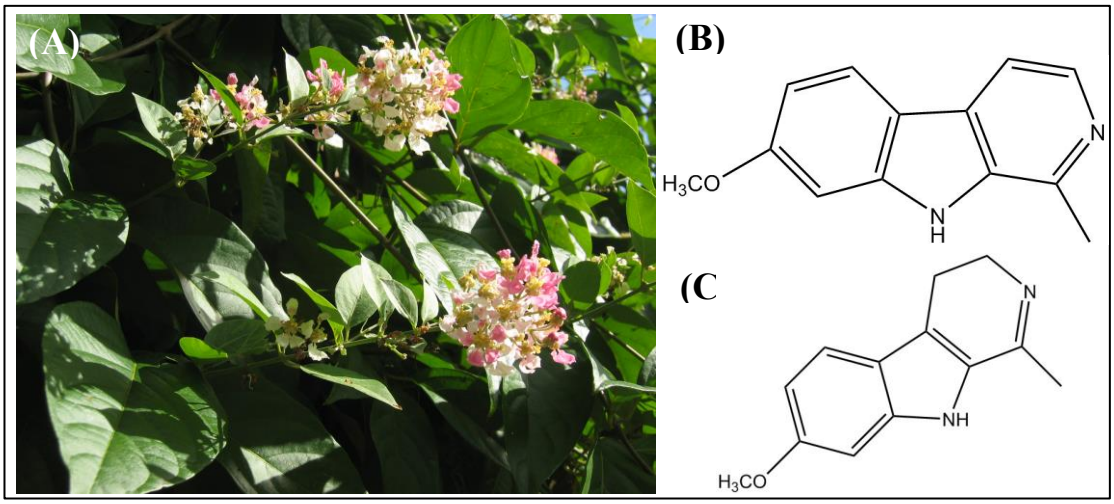


Figure 1.12. (A) Photograph of Ayahuasca (*Banisteriopsis caapi*) an Amazon native species by David Lorence (Font: National Tropical Botanical Gardens), **(B)** Harmine, and **(C)** Harmaline.

Some species of mushroom present psychoactive compounds as well. *Amanita muscaria* also known as Fly Agaric, has been used throughout history for medicinal, ritual and recreational purposes (Figure 1.13). Alkaloids like ibotenic acid and muscimol are the main constituents responsible for central nervous system activity. Furthermore, *A. muscaria* also contains muscarine, a potent cholinergic agonist (Michelot & Melendez-Howell, 2003; Halpern, 2004; Arunotayanun & Gibbons, 2012).

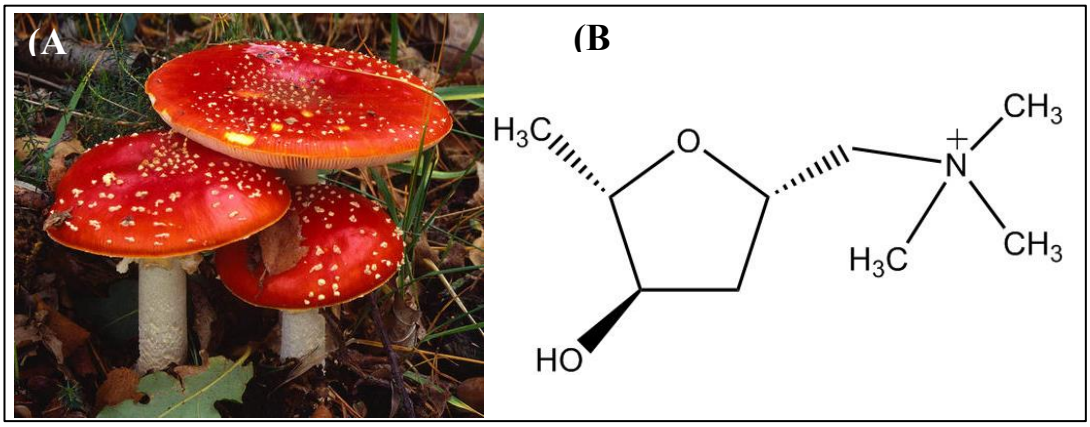


Figure 1.13. (A) Photograph by Geoffrey Kibby (Kew Royal Botanical Gardens) of *Amanita muscaria*, also known as Fly Agaric, **(B)** muscarine, the main psychoactive compound.

Salvia divinorum (Lamiaceae) is one of the most consumed legal highs in the UK. This plant is a Mexican native species, which the key psychoactive compound is a non-nitrogenous neoclerodane diterpene named salvinorin A (Figure 1.14). It is believed that its hallucinogenic effect is due to its selective k-opioid receptor agonist properties (Siebert, 1994; Arunotayanun & Gibbons, 2012).

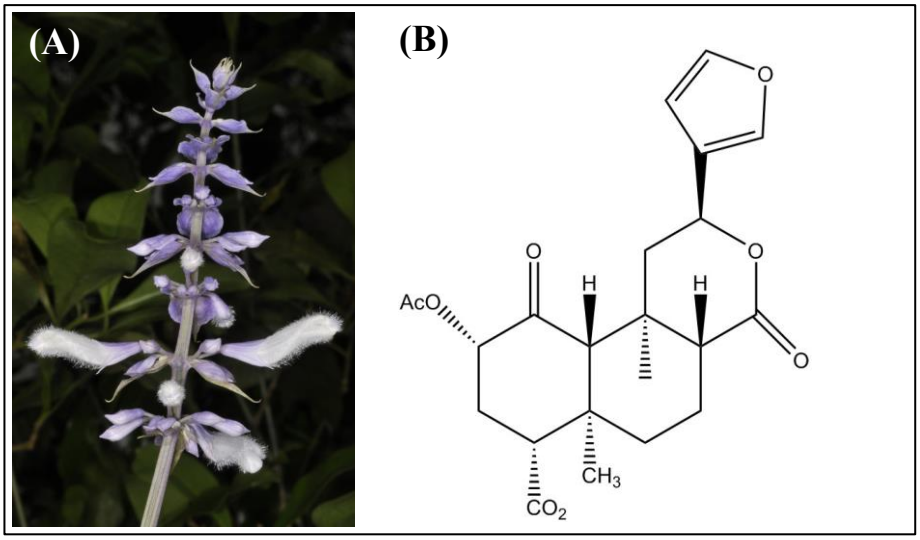


Figure 1.14. (A) *Salvia divinorum* (Font: Zurich Botanical Gardens / Botanischer Garten Zürich), **(B)** salvinorin A, the key compound responsible for the psychoactive properties.

Leonotis leonurus (Lamiaceae) is a robust shrub widespread throughout South Africa (Figure 1.15). The plant is widely used in oriental medicine and reports showed the presence of anticonvulsant, anti-nociceptive, anti-inflammatory and hypoglycemic activities. Some countries consider this species a legal high due to the orange colored flowers that are reported to have hallucinogenic properties (Agnihotri et al., 2009).

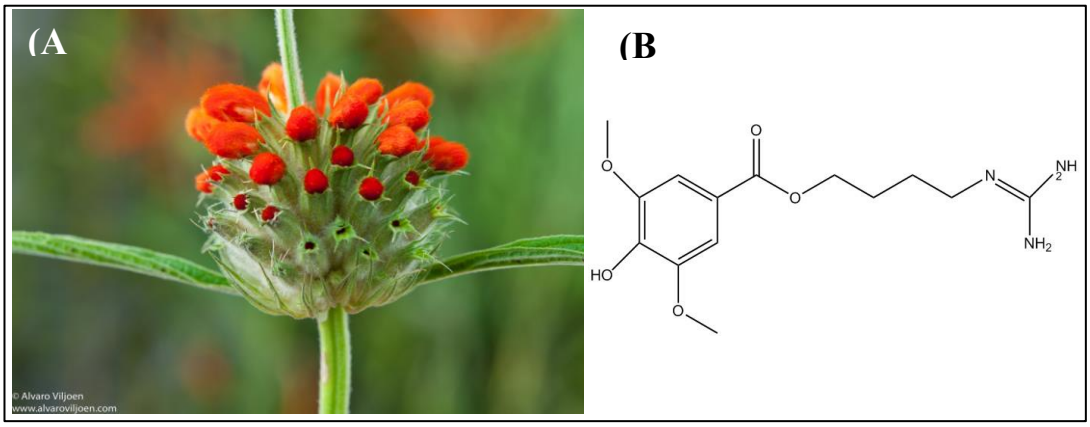


Figure 1.15. (A) Photograph of *Leonotis leonurus* by Alvaro Villjoen (Font: Tshwane University of Technology), **(B)** leonurine, the main compound present in this species.

1.4 LY2183240 Regioisomers

LY2183240 (*N*,*N*-dimethyl-5-[(4-biphenyl)methyl] tetrazole–1 carboxamide) is a novel compound synthesized by Ely Lilly and Company. This molecule can be

considered as a cannabinoid-type new psychoactive substance despite its chemical structure and mechanism of action on the cannabinoid system being different from those of other synthetic cannabamimetics on the market. Contrasting the emerging synthetic cannabinoids that act as direct agonists to cannabinoid receptors, LY2183240 elevates endogenous cannabinoid levels by disrupting the cellular uptake of a principal endocannabinoid, anandamide and arachidonoylglycerol, as well as inhibiting the main endocannabinoid degrading enzymes (Moore et al., 2005). Anandamide is a naturally occurring amide of arachidonic acid with ethanolamine, which it is released on demand by stimulated neurons and plays a significant role in both central and peripheral nervous system, becoming a useful therapeutic approach for pain, inflammation, insomnia, depression and anxiety management (Devane et al., 1992; Di Marzo et al., 1994; Piomelli et al., 1999; Kathuria et al., 2002).

Initial studies reported in literature on LY2183240 aimed to investigate the ability of this compound to inhibit the reuptake of anandamide. A study using a rodent model confirmed the analgesic property of LY2183240 without alteration in behaviour or motor responses of the subjects, unlike other cannabinoid receptor agonists, which generally cause undesirable adverse effects (Moore et al., 2005).

Moreover, LY2183240 was reported as a potent irreversible inhibitor of fatty acid amide hydrolase (FAAH), the key enzyme involved in the degradation of anandamide. Regarding the mechanism of action, it is believed that LY2183240 inactivates FAAH by covalently binding to the enzyme's serine nucleophile (Figure 1.16) (Alexander & Cravatt, 2006). Curiously, the test compound in this particular study was discovered later to be a mixture of LY2183240, 1,5 disubstituted carbamoyl tetrazole and its 2,5 regioisomer (Figure 1.17). Following studies reported that 2,5-LY2183240 behaves similarly to its isomer, but with relatively inferior pharmacological effect (Ortar et al., 2007; Ortar et al., 2008; Asada et al., 2015). The only difference between them is the position of the carbamoyl group in the tetrazole. Ortar et al. (2007) demonstrated that apart from the high potency against FAAH, 1,5-LY2183240 showed a moderate activity towards a monoacylglycerol lipase (MAGL), a degradative enzyme of another main endocannabinoid, 2-arachidonoylglycerol while 2,5-LY2183240 proved to be two times as active against MAGL than FAAH (Ortar et al., 2007; Ortar et al., 2008; Zvonok et al., 2008; Holtfrerich et al., 2013).

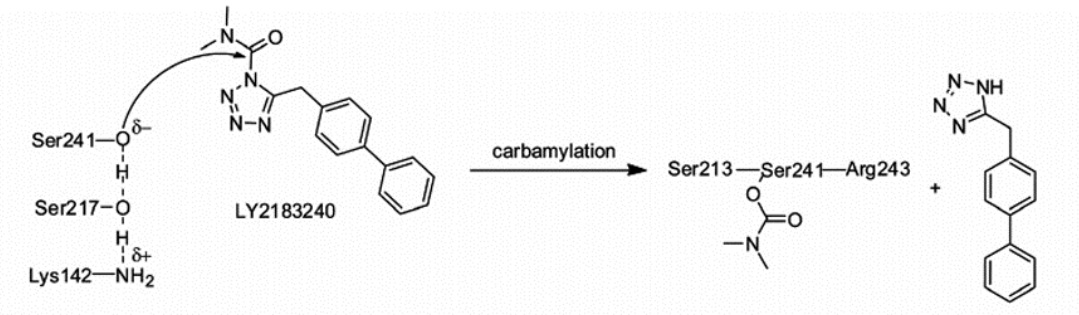


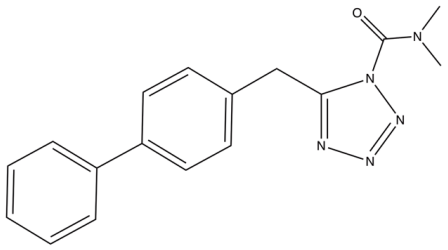
Figure 1.16 Predicted mode of irreversible inactivation of FAAH by LY2183240, involving carbamylation of the enzyme's serine nucleophile (Ser^{241}) (Alexander & Cravatt, 2006).

Intriguingly, Alexander and Cravatt (2006) revealed the identification of several additional serine hydrolases that were also inactivated by LY2183240 using functional proteomic screens. This finding indicates that this compound possesses rather promiscuous activity against this large and diverse enzyme class, becoming a relevant subject of study in many fields involving hydrolases.

More recently, both regioisomers were detected in herbal blend products as designer drugs in Japan. Concomitantly, two analogues of LY2183240 were also identified (Asada et al., 2015).

There have only been limited studies conducted on LY2183240 to date, especially regarding prokaryotic systems. This study aimed to explore the antimicrobial properties of the regioisomers in detail.

(A)



(B)

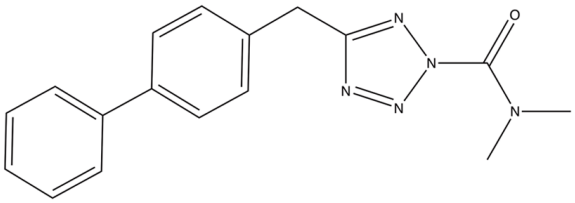


Figure 1.17. Chemical structures of the LY2183240 regioisomers design by ChemDraw® software version 16 **(A)** 1,5-LY2183240; **(B)** 2,5-LY2183240.

2 CHAPTER 2

Materials and Methods

2.1 Materials

2.2 Plant Materials

Plant species, namely *Trichocereus peruvianus, Trichocereus pachanoi, Mitragyna speciosa, Argyreia nervosa, Banisteriopsis caapi, Salvia divinorum, Amanita muscaria,* and Morning Glory seeds, were purchased on-line from various websites (listed in Table 2), with the exception of *Leonotis leonurus,* which was collected in the Shannon, South Africa, and kindly donated for this research project.

Table 2. The source of the legal highs samples tested.

Sample	Website	Quantity
***Salvia divinorum* leaves**	http://partyherbals.co.uk/index.php/	3.5g
Argyreia nervosa	http://partyherbals.co.uk/index.php/	10g
Banisteriopsis caapi	http://www.avalonmagicplants.com/	10g
Mitragyna speciosa	http://azarius.net/smartshop/	25g
Trichocereus peruvianus	http://azarius.net/smartshop/	8g
Amanita muscaria	http://salviaonline.co.uk/	3g
Trichocereus pachanoi	http://salviaonline.co.uk/	12.5g
Morning Glory seeds	http://salviaonline.co.uk/	25g

2.2.1.1 Chemical Material

During the screening phase of this study, synthetic compound LY2183240, purchased from the website "*Buy Any Chem*" at the average price of £ 10.00 per 500 mg (2013), was also investigated.

The 1,5-regioisomer of LY2183240 5-([1,1'-biphenyl]-4-ylmethyl)-*N*,*N*-dimethyl-1*H*-tetrazole-1-carboxamide (Figure 1.17A) was obtained from Santa Cruz Biotechnology, Inc., Dallas, Texas, USA and 2,5-regioisomer of LY2183240 5-([1,1'-biphenyl]-4-ylmethyl)-*N*,*N*-dimethyl-2*H*-tetrazole-1-carboxamide (Figure 1.17B) from Cayman Chemical, Ann Arbor, Michigan, USA. Both compounds were stored at -20°C, protected from light and moisture.

2.2.2 Bacteria

Table 3. The microorganisms tested.

Strain	Gram	Source	Antibiotic resistance	Note
S. aureus 12981	+	NCTC	MSSA	Control Strain
S. aureus 13373	+	NCTC	MRSA	Standard Strain
MRSA 80415G	+	Clinical Isolate	MRSA	-
MRSA 80415R	+	Clinical Isolate	MRSA	-
MRSA 80415S	+	Clinical Isolate	MRSA	-
MRSA 80415K	+	Clinical Isolate	MRSA	-
EMRSA 15	+	NCTC	MRSA	-
S. aureus 1199b	+	ATCC	Norfloxacin	NorA efflux pump
S. aureus RN4220	+	Clinical Isolate	MRSA	From *S. aureus* 8325
S. aureus XU212	+	(Gibbons & Udo, 2000)	Tetracycline	TetK efflux pump
S. epidermidis 12228	+	ATCC	MSSA	Standard Strain
S. pneumoniae 12695	+	NCTC		Standard Strain
B. subtilis 15	+	NCTC	Susceptible	Control Strain
E. faecalis 12967	+	NCTC	Susceptible	Control Strain
E. faecalis 13379	+	NCTC	Gentamicin	Standard Strain
Stenotrophomonas sp.	-	NCTC	Susceptible	Standard Strain
E. coli 10418	-	NCTC	Susceptible	Control Strain
E. coli G69	-	Clinical Isolate	MDR	-
C. freundii 382010	-	NCTC	Susceptible	Standard Strain
K. pneumoniae 17	-	NCTC	MDR	-
P. aeruginosa 10662	-	NCTC	Susceptible	-
Micrococcus lysodeikticus 4698	-	ATCC	Susceptible	Standard strain

2.3 Methods

2.3.1 Ultrasound-Assisted Extraction of Plant Material

The plant materials were pulverised into fine powder with a blender. Ten grams (10 g) of each powder of the plant was macerated in 100 mL of different solvents: *n*-hexane, ethyl acetate and methanol. Extracts were prepared by an ultrasound-assisted

process for 1h at room temperature and filtered through Whatman No. 2 filter paper. Filtrates obtained from each extraction were exposed to nitrogen gas to remove any residual solvent, except the methanol extract, which was concentrated under vacuum (Buchi R-114, Switzerland). The extracts obtained were stored at 4°C until further use.

Twenty-five grams of *L. leonurus* was extracted with 250 mL of *n*-hexane, chloroform, methanol and dichloromethane in sequence, by use of a Soxhlet apparatus. The extract was filtered through Whatman paper filter No. 2, concentrated at 55 ± 3°C under reduced pressure. Again, the extracts obtained were preserved at 4°C prior to use.

2.3.2 Chemical Characterization

In this study, the chemical characterisation was performed only on samples that previously revealed antimicrobial activity. Various types of techniques were used including Thin Layer Chromatography and High Performance Liquid Chromatography, as well as spectroscopic approaches like Nuclear Magnetic Resonance, and Mass Spectroscopy.

2.3.2.1 Chromatographic Techniques

2.3.2.1.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was performed on LY2183240 to assess the purity of the compound purchased. The LY2183240 powder was dissolved in methanol (1 mg/mL) and placed on a 10 x 10 cm silica gel TLC plate (Sigma Aldrich, UK). The mobile system was developed with hexane: ethyl acetate (30:70) and UV light (254 nm and 366 nm) was used for visualization of the bands in the plate.

2.3.2.1.2 High Performance Liquid Chromatography (HPLC)

HPLC is a sophisticated technique of liquid chromatography used to separate the intricate mixture of molecules in both chemical and biological systems, offering a combination of speed, reproducibility and sensitivity (McMaster, 2007).

After the TLC test, further characterisation of samples of LY2183240 (mixture, -1,5 and -2,5) were performed by HPLC with a Agilent 1200 series instrument supplied

with a binary solvent manager, sample manager and photodiode array detector (PDA) coupled with a Q-TOF mass spectrometer. An XTerra column, 2.5 μm particle size, 4.6 x 50 mm (Waters, Milford, MA, United States) was utilized at a temperature of 40°C with a flow rate of 1 mL/min. The gradient system is summarized in Table 4.

Table 4. Gradient system used in HPLC analysis.

Time (minutes)	**Solvent A** 0.1% formic acid	**Solvent B** 0.1% formic acid in acetonitrile
1	90%	10%
8	5%	95%
10	5%	95%
10.2	90%	10%
12	**Stop**	

2.3.2.2 Spectroscopic Methods

2.3.2.2.1 Nuclear Magnetic Resonance (NMR) Analysis

Samples of LY2183240 (5 mg) were dissolved in 0.7 mL methanol-d_4, transferred to 5 mm NMR tubes and subjected to NMR spectroscopy analysis. NMR experiments were performed on a Bruker Avance 400 spectrometer operating at 400 MHz (Bruker BioSpin) for one-dimensional (^{1}H, ^{13}C and DEPT), as well as two-dimensional (COSY, HMQC, HMBC and NOESY) analysis. The data generated were processed and visualized by the software TopSpin v2.1. Chemical shifts were presented in part per million (ppm), which were generally scaled from 0 to 15 ppm for ^{1}H-NMR spectra and from 0 to 220 ppm for ^{13}C NMR spectra, while coupling constant (*J* values) were reported in Hertz (Hz).

2.3.2.2.2 Mass Spectrometry Analysis

Samples of LY2183240, including regioisomers -1,5 and -2,5, were dissolved in acetonitrile (100 %) to reach a concentration of 5 mg/mL, transferred to a glass vial and submitted to analysis.

Mass determinations were conducted by the structural analysis team at the UCL School of Pharmacy. Electrospray ionization mass spectrometry (ESI-MS) was performed on a Finnigan Navigator instrument. The mass spectra were plotted as arrays of vertical lines (ion peaks) of relative abundance of the ions in % (x-axis) against the ratio of mass-to-charge (*m/z*) value (y-axis). The highest peak represented the most abundant ion which typically was the most stable ion generated during the ionization process.

2.3.3 Biological Evaluation

2.3.3.1 Minimum Inhibitory Concentration (MIC) Assay

The antibacterial activity of the extracts and synthetic compound LY2183240 were tested by the broth microdilution assay according to Andrews (2001). Briefly, all bacterial strains were cultured on nutrient agar (Sigma-Aldrich, UK) plates and incubated for 24 hours at 37°C prior to MIC determination. In addition, known quantities of each test sample were dissolved in DMSO and then diluted in ISB (Iso-Sensitest Broth, Oxoid, UK) to give a range concentration of 512 – 0 µg/ml, unless stated differently. The DMSO concentrations employed in all experiments showed no inhibitory effect towards bacterial growth. Finally, the overnight cultures of each of the tested strains were suspended to an inoculum density of approximately 1.0×10^8 CFU/mL in the Phosphate Buffered Saline (PBS), consisting of 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 15 mM KH_2PO_4 (Oxoid, UK). The cell suspensions were standardized by adjusting the optical density to 0.1 at 600 nm (Thermo Scientific UV-Vis Spectrophotometer, UK) before being diluted 1:100 in ISB prior to inoculation. Antimicrobial agents such as penicillin, norfloxacin, and amoxicillin were used as positive controls for the assay.

The assays were performed by microdilution using 96-well microtiter plates with a final inoculum of $5x10^5$ CFU/mL and each sample was tested in duplicate in at least two independent experiments in order to confirm the reliability of the data. Results were determined by visual inspection of the wells and the presence of an evident opaque medium or white pellets were indicative of bacterial growth. The MIC values were recorded as the lowest concentration at which no bacterial growth was detected (Figure 2.1).

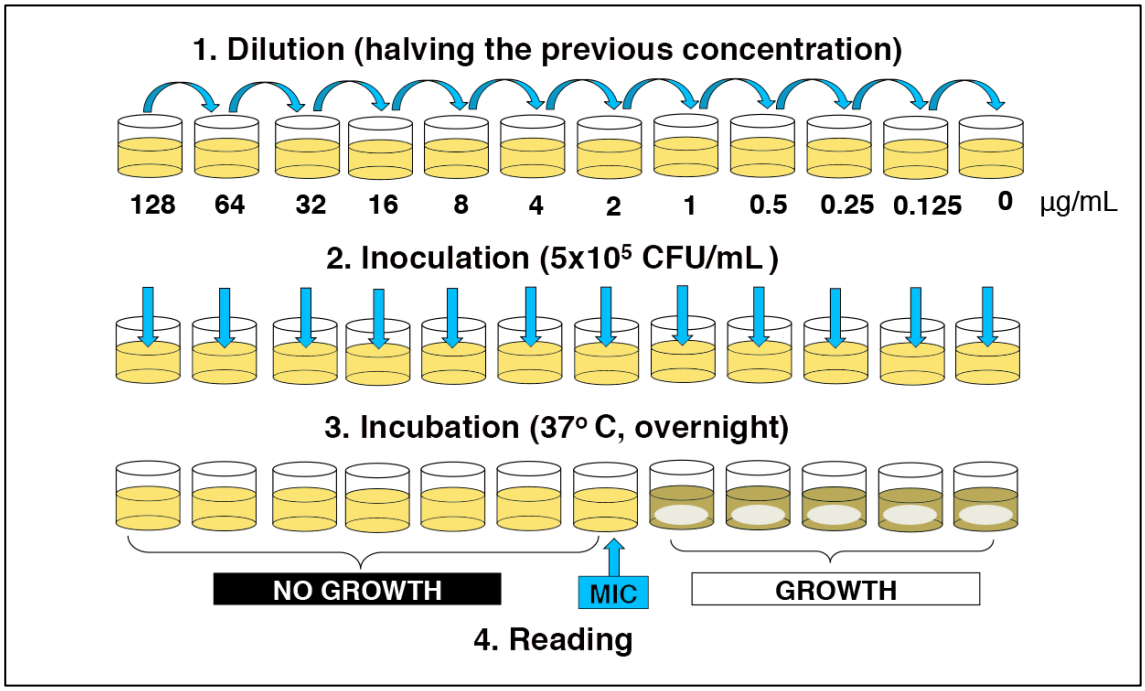


Figure 2.1. Representative illustration of the microbroth dilution technique.

2.3.3.2 Minimum Bactericidal Concentration (MBC) Assay

The minimum bactericidal concentration (MBC) is the lowest concentration of an antimicrobial agent necessary to reduce the viability of the initial bacterial inoculum by ≥ 99.9 %. The MBC along with the MIC assist in the determination of the mechanism of action of the antimicrobial agents, e.g., bacteriostatic or bactericidal effect.

After MIC determination, the wells showing no growth were sub-cultured onto nutrient agar and incubated overnight at 37°C to determinate the number of viable CFU present (Lorian, 2005).

2.3.3.3 Antimicrobial Potentiation Assay

To assess the antibiotic resistance modifying activity of the extracts and synthetic compounds, the MICs of antibiotics were determined in the presence or absence of the test samples using the broth microdilution technique as aforementioned (Andrews, 2001). All bacterial strains were cultured on nutrient agar for 24 h at 37°C prior to MIC determination. The test samples (modulators) and antimicrobial agents were prepared by dissolving in DMSO. The concentration of the modulators was

fixed throughout the experiment whilst antimicrobial agents were serially diluted across the 96-well microtiter plates. The MIC of the antimicrobial agents was determined in combination with sub-inhibitory concentrations of the modulators.

2.3.3.4 Bioautographic Agar Overlay Method

Bioautography has been considered one of the most effective procedures for the detection of antimicrobial agents, because it permits the location of the activity especially in a complex matrix, hence allows a target-directed isolation of the active components (Rahalison et al., 1991).

The bioautographic agar overlay method was performed based on the procedure of Hamburger & Cordell (1987), with modifications. Firstly, the TLC plates (10 × 10 cm) containing LY2183240 bands were developed as described in section 2.3.2.1.1. Afterwards the TLC plates were carefully cleaned with ethanol and placed into a square Petri dish (Thermo Scientific, UK). A suspension of *S. aureus* 12981 to achieve a final inoculum of 5 x 10^5 CFU mL^{-1} was added to 200 mL of molten nutrient agar. A sample of around 25 mL of the inoculated nutrient agar was dispersed evenly over the TLC plate. The plates were incubated overnight at 37°C. Clear inhibition zones observed against a black background indicated an active compound.

2.3.3.5 Inhibition Zone Determination by Disc Diffusion Assay

The antimicrobial activity of LY2183240 was also assessed by a disc diffusion method. Briefly, LY2183240 was dissolved in dimethyl sulphoxide (DMSO, Merck, Germany). Iso-Sensitest Agar (Oxoid, UK) was prepared and autoclaved at 121°C for 20 minutes. Twenty millilitres of the sterilised agar medium were poured into sterile Petri dishes under aseptic conditions and allowed to set.

Overnight cultures of *S. aureus* 12981 and *B. subtilis* were prepared for the inocula and suspended in PBS. The cell suspensions were standardized by adjusting the optical density to 0.1 at 600 nm (Thermo Scientific UV-Visible Spectrophotometer, UK). One hundred (100 μL) microlitres of cell suspension with approximately 10^8 CFU/mL were placed in Petri dishes and dispersed over the agar. Hereafter, sterile paper discs were saturated with 10 μL of the different compounds extracted from the

LY2183240 mixture and allowed to dry at 37°C for 1 hour before being placed onto the agar. A disc prepared under the same conditions containing DMSO was used as a solvent control (Tekwu et al. 2012).

2.3.3.6 Plasmid Conjugation Assay

The broth-mating for *E. coli* assay employed in this study was described by Rice and Bonomo (2005) with modifications. McConkey agar and LB broth were prepared and autoclaved. The appropriate antibacterial agents were added to McConkey agar depending on the plasmid tested (Table 5).

Table 5 Antibiotics used for the preparation of donor and transconjugant selection plates.

Test plasmid	Agar plates for donor growth	Agar plates for transconjugant growth
TP114	kanamycin (10 μg/ml)	kanamycin (10 μg/mL) + streptomycin (20 μg/mL)
pKM101	amoxicillin (30 μg/ml)	amoxicillin (30 μg/mL) + streptomycin (20 μg/mL)
pUB307	kanamycin (30 μg/ml)	kanamycin (30 μg/mL) + streptomycin (20 μg/mL).
R7K	amoxicillin (30 μg/ml)	amoxicillin (30 μg/mL) + nalidixic acid (30 μg/mL)

In the mating experiment, the donor and a recipient strain carrying different resistant determinants are mixed together. Subsequently, the transconjugants were selected on a selective agar plate containing two antimicrobial agents. It is possible to apply selective conditions that inhibit both the recipient and the donor and allow the growth only of the recipients that acquired the transferable plasmid from the donor (transconjugants). In this context, the addition of LY2183240 in the mating broth evaluated the compound's inhibitory activity against plasmid transfer, in comparison with a drug free control. Two or three colonies of an overnight culture of each strain were inoculated to different flasks containing 5 mL of LB broth and incubated for 16 h at 37°C. This was followed by the mating of the strains, which included the addition of 450 μL of PBS, 250 μL of a donor, 250 μL of the recipient and 2.5 mL of

the test sample or in LB broth alone (control) in the same flask. The flask containing the different combinations of donors and test samples were incubated overnight at 37°C. A serial dilution of the mixture were prepared and plated to the selective agar plates. All plates were incubated overnight at 37°C and eventually the colonies grown on each plate were counted and the transfer frequency in the presence and absence of LY2183240 was determined. The transfer frequency was calculated as the number of transconjugant, to the number of the donor colonies, both expressed as CFU/mL.

2.3.3.7 Bacterial Cell Morphology Assay

The goal of this experiment was to identify potential target or targets of LY2183240 in Gram-positive strains, especially *B. subtilis* and *S. aureus*. Consequently, due to the capacity of LY2183240 to inhibit eukaryotic hydrolases, an assessment of the effect of the agent on bacterial hydrolases was performed.

The process of cell division involves the association of a large number of proteins at the mid-point of the cell. These enzymes catalyze the separation of nucleoids, the formation of a septum, and the division of the cell into two daughter cells (Robichon et al., 2008). Bacterial peptidoglycan hydrolases constitute an ample and immensely diverse group of enzymes, those whose main functions include the regulation of cell wall growth, the turnover of peptidoglycan during growth, the separation of daughter cells during cell division and autolysis (Vollmer et al., 2008).

The effect of LY2183240 mixture on cell morphology was examined over time and compared to the effect that the antimicrobial agent cefotaxime exhibits on bacterial cell shape. Inocula were incubated to reach the appropriate optical density at 600 nm. 100 µL of *S. aureus* 12981 or *Bacillus subtilis* 13 were then added to 900 µL ISB containing various concentrations of cefotaxime (0.01, 0.1 and 1 µg/mL) or LY2183240 (16, 160 and 1.6 µg/mL). The resulting solutions were left in an incubator-shaker (GenLab) for 90 minutes at 37°C. Around 0.5 µL of the cell suspensions were spread onto a glass slide, 76 x 26 mm (Thermo Scientific, UK) and fixed by passing through a Bunsen flame twice, and then examined with the aid of a phase-contrast microscope (Zeiss light imaging, Axio Cam MR). Sequential photos were taken, using Zen Pro 2012 software. Between time points the solutions were

left in the incubator to allow continuation of the exponential growth of the cells. Control experiments in the absence of cefotaxime or LY2183240 were performed for comparison.

2.3.3.8 Modulation of Penicillin-Induced Cell Lysis

The ability of LY2183240 to inhibit cell lysis was assessed with penicillin G as the cell lysing-inducing agent. A *M. lysodeikticus* ATCC 4698 (Sigma – Aldrich, UK) suspension was prepared in PBS (0.1% w/v) and mixed with penicillin G (128 to 0.125 µg/mL) or LY2183240 (800 to 12.5 µg/mL). The optical density (600nm) was measured in a microplate spectrophotometer (BioTek, UK) at 37°C, over a period of 120 minutes at 5 minute intervals.

2.3.3.9 Modulation of Triton X-100-Induced Cell Lysis

Assessment of the capacity of LY2183240 to affect Triton X-100-induced cell lysis was accomplished by the method of Mani et al. (1993), with modifications. Briefly, 5 mL cultures of *S. aureus* 12981 (MSSA) growing exponentially in ISB medium were collected by centrifugation (8,000 × *g*, 4°C, 15 min). Cells were washed twice with 5 mL of cold distilled water and re-suspended in 5 mL of 0.05 M Tris-HCl (pH 7.4) containing 0.05 % (v/v) Triton X-100 (Sigma-Aldrich, UK). The cells were incubated at 37°C with shaking and the absorbance was measured (630 nm) at 30-min intervals for 3 h.

2.3.3.10 Inhibition of Lysozyme

The inhibitory activity of LY2183240 mixture against lysozyme (EC 3.2.1.17, Sigma-Aldrich, UK) was assessed by a cell lysis assay employing a cell suspension of *M. lysodeikticus* ATCC 4698 in PBS, pH 7.2. The cell suspension was mixed with PBS containing 400 units/mL of lysozyme and LY2183240 mixture. The concentration of LY2183240 was 170 mM. Imidazole (Sigma – Aldrich, UK) was used as a positive control of lysozyme inhibition at the same concentration.

The reaction mix was incubated at 37°C and lysozyme activity was quantified by measuring the absorbance (630 nm) at different times with a 96-well microtiter plate

spectrophotometer (Biotek, UK) with plate shaking function. Analytical data were processed by Gen5 v1.07 software.

2.3.3.11 Detection of Lytic Activity in SDS-PAGE Gels

The lytic activity of enzyme extracts was determined as described by Mani et al. (1993). Briefly, a 10 % polyacrylamide-sodium dodecyl sulfate (SDS) gel containing 0.1 % (w/v) crude cell walls from *S. aureus* 12697 was prepared. Purified cell walls and crude cell walls were prepared from exponential-phase cultures of *S. aureus* strains grown in LB medium as described by Jayaswal et al. (1990). The enzyme extracts were mixed with loading buffer (control) and 2,5-LY2183240 (128 µg/ml). Imidazole was used as positive control at the same concentration. Samples were heated at 98°C for 3 min in a thermo cycler (Fisher Scientific, UK) prior to polyacrylamide gel electrophoresis (PAGE). After electrophoresis the gels were incubated for 12 to 16 h at 37°C in 25 mM Tris-HCl (pH 7.4) containing 1 % Triton X-100 to allow protein renaturation. Bands with lytic activity were observed as clear zones in the opaque gel. Gels were stained with 1 % methylene blue in 0.01% KOH prior to photography. For total protein profile analysis, SDS-15% PAGE gels were used, and bands were visualized by staining with coomassie blue dye.

2.3.4 *In Vitro* Protein Synthesis Assay

The *in vitro* protein synthesis experiments were performed using the commercially available cell-free transcription-translation system composed of purified components PURExpress (New England BioLabs Inc., UK). The protocol was followed according with the manufacturer instructions using a template DNA of dihydrofolate reductase (DHFR). To evaluate the potential protein synthesis inhibition, 128 µg/ml of 2,5-LY2183240 was added into the reaction. A positive control using chloramphenicol was performed.

After *in vitro* transcription/translation, reactions could be analyzed by SDS-PAGE followed by staining with coomassie blue dye.

2.3.5 Effect of Cell Membrane Permeabiliser, PEI, on the Anti-Gram-Negative Activity of 2,5-LY2183240

Gram-negative bacteria typically are enclosed by an outer membrane (Nikaido, 2003). This barrier protects bacteria against austere environments and consequently have a major influence on the susceptibility to external agents such as antibiotics, which, in most of cases, are targeted at intracellular processes (Koebnik et al., 2000; Delcour, 2009).

In this respect, the main objective of this assay was to evaluate the effect of the 2,5-regioisomer towards Gram-negative bacteria in the presence of the permeabilising agent polyethyleneimine (PEI).

The susceptibility of *E. coli* 10418 to 2,5-regioisomer was tested by broth microdilution procedure with and without PEI supplementation (at 256 μg/mL). Novobiocin was used as a standard reference under the same conditions. Likewise, polyethyleneimine was evaluated alone and in combination with novobiocin.

2.3.6 Wall Teichoic Acid (WTA) Extraction

Bacteria are surrounded by a complex cell envelope that performs a variety of functions (Silhavy et al., 2010). In many Gram-positive bacteria, the peptidoglycan layers are densely functionalized with anionic glycopolymers called wall teichoic acids (WTAs). These polymers play vital roles in cell shape determination, regulation of cell division, as well as other fundamental aspects of cell physiology. Moreover, WTAs are important in the pathogenesis of infection and play key roles in antibiotic resistance (D'Elia et al., 2006; Atilano et al., 2011; Brown et al., 2013).

In this respect, the primary goal of this assay is to examine if the biosynthesis of staphylococcal wall teichoic acid could be a potential target of 2,5-LY2183240.

The assay was divided into two parts, namely WTA extraction and WTA PAGE analysis.

2.3.6.1 Wall Teichoic Acid Extraction

The extraction of the wall teichoic acid was conducted according to Meredith et al. (2008) and Endl et al. (1983), with modifications. Briefly, WTA was extracted from a 20-mL culture of *S. aureus* NCTC 12981 grown in ISB overnight at 37°C. The

cells were collected by centrifugation (5,000 × g) for 10 min. Afterwards, they were subjected to washing with 30 mL of buffer 1 [50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 6.5], and re-suspended in 30 mL of buffer 2 (4 % [wt/vol] sodium dodecyl sulfate (SDS), 50 mM MES; pH 6.5). Samples were boiled for 1 h, and the cells collected by centrifugation (8,000 × g) for 10 min. The pellet was re-suspended in buffer 2, transferred to a 1.5-ml eppendorf tube, and sedimented at 14,000 × g for 10 min. Subsequently, the pellet was washed once with buffer 2, one more time with buffer 3 (2% NaCl, 50 mM MES, pH 6.5), and finally with buffer 1. After the last wash, samples were treated with proteinase K (20 mM Tris-HCl, pH 8.0; 0.5% wt/vol SDS, and 20 μg of proteinase K in 1 mL) and incubated at 50°C for approximately 4 hours. Following digestion, samples were washed once with buffer 3 and then at least three times with distilled water to remove the SDS. Samples were carefully re-suspended in 1 mL of 0.1 M NaOH and shaken at room temperature for 16 h to hydrolyse the WTAs. Insoluble cell wall debris was removed by centrifugation (14,000 × g, 10 min), and the supernatant containing the hydrolysed WTA was directly analysed by polyacrylamide gel electrophoresis (PAGE).

2.3.6.2 Wall Teichoic Acid PAGE Analysis

The WTA PAGE technique was performed according to Pollack & Neuhaus (1994) and Meredith et al. (2008). Electrophoresis was performed utilising a Mini-Protean Tetra cell (Bio-Rad, Hercules, California, USA) at a constant current of 80 mA to achieve separation.

WTA samples were diluted 1:3 in loading buffer (Sigma-Aldrich, UK) to obtain a final volume of 10 μL and loaded in a 20%-gel (total acrylamide). Gels were developed at ~4°C for about 18 hours using the running buffer (0.25 M Tris-base, 1.92 M glycine, and 1 % SDS w/v). WTA bands were visualized using a coomassie blue staining protocol (Bio-Rad, Hercules, California, USA).

2.3.6.3 Lipoteichoic Acid (LTA) Potential Target Analysis

The main objective of this assay was to verify if the synthesis of lipoteichoic acid is a potential target of 2,5-LY2183240 anti-staphylococcal activity. An agar well diffusion test was performed according to Magaldi et al. (2004). Briefly, 2,5-LY2183240 (5 µg/mL) was added to a well containing 100 µg of LTA. Two controls containing only the regioisomer and LTA were also prepared.

2.3.7 Fatty Acid Biosynthesis Inhibition Assay

MICs were determined in accordance with the broth microdilution technique (section 3.3.7). In addition, a filtered solution of 0.1 % Tween 80 was supplemented to wells containing the 2,5-LY2183240 (0 – 128 µg/mL) and Triclosan (0 – 32 µg/mL). Modulation of activity was defined as a greater than or equal to a 10-fold decrease in CFU mL^{-1} for the combination compared to the agent alone. All the experiments were conducted in triplicate.

2.3.8 Selecting Drug-Resistant Mutants

The traditional and classic technique of selecting mutants capable to grow in the presence of a toxic concentration of a novel identified compound continues to be a useful target identification strategy (Farha & Brown, 2016).
A paper-disc diffusion method was used to select the drug-resistant mutants. A high-density culture of *S. aureus* 12981 was applied on the agar plate and left to dry. Afterwards, 100 µg/ml of 2,5-LY2183240 and 10 µg/ml of triclosan were added on paper discs and fixed on the agar. The plates were incubated for 48 hours at 37°C.

2.3.9 DNA Extraction and PCR Amplification of the **fabI** *Gene*

DNA extraction was performed using DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany) and the protocol was followed according to manufacturer instructions. PCR amplification of the *fabI* genes of *S. aureus* drug-susceptible and -resistant mutants were performed with primers:

5'–CGGACGATACAGAGTTAGTTGATTC–3'

5'–GCTAAATTTTTCAAAGGTGAACGTA–3'

Amplification primers were designed based on the extracted sequence 919,754 (1) to 920,994 (1,241) from *Staphylococcus aureus* subsp. aureus NCTC 8325 complete genome (accession number CP000253). Primers were identified with Primer3 (default settings); target sequence was 919,873 (120) to 920,853 (1,100 bp). PCR amplification conditions were: 1 min at 98ºC for 1 cycle; 30 sec at 98ºC, 30 sec at 65ºC and 1 min at 72ºC for 30 cycles; and 10 minutes at 72ºC for 1 cycle. The enzyme used was Phusion High-Fidelity DNA polymerase (NEB, UK). The PCR products (1103 bp) were purified utilizing a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sent for sequencing at UCL Sequencing Facility. The data were processed using Geneious software version 6.1.6.

2.3.9.1 Western Blotting of FabI Expression

The level of FabI protein expression in *S. aureus* strains was quantified by western blotting by means of polyclonal rabbit antibodies raised against recombinant *S. aureus* enoyl-[acyl-carrier-protein] reductase [NADPH] FabI protein (LifeSpan BioSciences, Inc., Seattle, USA).

Bacterial protein extraction was performed using B-PER™ reagents (ThermoFisher, UK) with the addition of 200 units of lysostaphin (Sigma-Aldrich, UK). Briefly, an exponentially growing culture of *S. aureus* was pelleted by centrifugation at 5000 × *g* for 10 minutes. 4 mL of B-PER reagent containing lysozyme (50mg/mL), DNase I (2500U/mL) and lysostaphin (200 U/mL) was added for each gram of cell pellet and incubated for 15 minutes at room temperature. Lastly, the lysates were centrifuged at 15,000 × *g* for 5 minutes to separate soluble proteins from the insoluble cell debris. The protein concentration in the lysates was determined by using a Bio-Rad DC kit in accordance with the manufacturer's instructions.

Western blot processing was performed with the WesternBreeze™ Chromogenic Kit, anti-rabbit (Thermo Scientific, UK), using anti-FabI as a primary antibody. MagicMark™ XP Western was used as a protein standard marker.

After running the SDS-PAGE gel, the proteins were transferred to a PVDF membrane. The membrane was then placed in 10 mL of the appropriate Blocking Solution in a covered, plastic dish and incubated for 30 minutes on a rotary shaker.

The Blocking Solution was then decanted and the membrane was rinsed with 20 mL of water for 5 minutes, twice. Afterwards, the membrane was incubated with 10 mL of Primary Antibody Solution for 1 hour, then decanted and washed for 5 minutes with 20 mL of prepared Antibody Wash, 3 times. Subsequently, the membrane was incubated in 10 mL of Secondary Antibody Solution for 30 minutes, and subjected to the same washing procedure as aforementioned. Lastly, the membrane was incubated with 5 mL of Chromogenic Substrate until purple bands developed on the membrane, at which point the membrane was rinsed and dried on a clean piece of filter paper.

2.3.10 β-Lactamase AmpC Purification

2.3.10.1 Affinity Chromatography

The purification of the AmpC β-lactamase from *Enterobacter cloacae* was performed by the method of Cartwright and Waley (1984) with modifications. A boronic acid column with a hydrophilic spacer arm (Figure 2.2) was prepared as follows. 20 mL of Affi-Gel 10 was washed with propan-2-ol and then water at 4°C and transferred to 20 mL of 1 M-$KHCO_3$ containing 2 g of *m*-aminophenylboronic acid hemisulphate and 2 g of sorbitol under agitation at room temperature for 1h. The gel was poured into a column 10 cm high x 2.5 cm diameter and washed consecutively with 1 M-NaCI/0.5M-sorbitol, pH 7 (200 mL), 0.5 M-borate, pH 7 (200 ml), and lastly 20 mM-triethanolamine hydrochloride/0.5 M-NaCl (loading buffer), pH 7.0. The column was then ready for use.

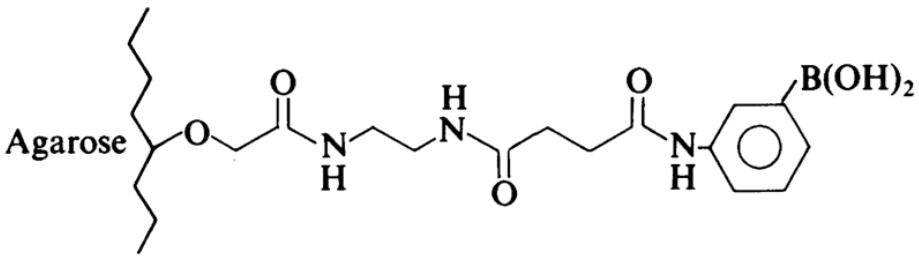


Figure 2.2. A typical boronic acid column with a hydrophilic spacer arm.

Crude protein extract containing 0.2 - 0.6 units/mg protein of β-lactamase P99 (Sigma-Aldrich, UK) was dissolved in loading buffer and ran through a column at a

flow rate of 30 mL/h. The column was washed with loading buffer until the OD_{280} of washings was zero. The AmpC β-lactamase was then eluted with 0.5 M-borate/0.5 M-NaCl, pH7. The columns were regenerated by washing with this borate buffer, stored at 4°C, and re-used repeatedly with no loss of binding capacity detected. The use of buffers of high ionic strength minimizes ionic interactions (Cartwright and Wiley, 1984). The samples were then analysed by SDS-PAGE electrophoresis.

2.3.10.2 SDS-PAGE Electrophoresis Analysis

SDS-PAGE is a consistent method for determining the purity and molecular weight of a particular protein; the migration position of a protein coated with SDS is inversely proportional to the logarithm of its molecular weight.
A 10 μL aliquot of each fraction was loaded with SDS sample buffer (New England BioLabs, UK) and heated at 98°C for 3 min in a thermocycler (Fisher Scientific, UK) before loading into a well of a 10 % acrylamide gel (16 cm x 14 cm x 3 mm). Electrophoresis was performed in a Mini-Protean Tetra cell using 130 V and 500 mA for the separation. The running buffers contained 0.25 M Tris-base, 1.92 M glycine, and 1% (w/v) SDS. Proteins were stained with coomassie blue (Bio-Rad) for 60 minutes with gentle shaking and subsequently washed with distilled water for 15 minutes at least 3 times.

2.3.10.3 Protein Concentration and Content Determination

The fractions containing a ~39-kDa protein, corresponding to the P99 β-lactamase, were pooled and concentrated with a Vivaspin 500 centrifugal concentrator (10 kDa MW cut-off; Vivascience, Hannover, Germany) in sterile-filtered PBS and total protein content determined by the Bradford method (Ramagli & Rodriguez, 1985). All extracts were combined and stored at – 20°C until needed.

2.3.10.4 Fast Protein Liquid Chromatography (FPLC)

Fast protein liquid chromatography (FPLC) is a frequently employed liquid chromatographic method for macromolecules in gel separation at low pressure and with high capacity. The adsorbents, composed within a gel matrix with functional

ligands, are one of the most essential features of this chromatography technique (Madadlou et al., 2011). Commonly, FPLC chromatography is performed on material that has already been exposed to some preliminary chromatographic steps. Gel filtration, for instance, is frequently used as a final step to eliminate minor impurities from a partially purified protein (Madadlou et al., 2011).

The FPLC gel filtration of the sample previously semi-purified was performed in 0.05 M KH_2PO_4 buffer (pH 5.0), at a flow rate of 1 mL/min and using a Superdex 75 column, which had been calibrated with molecular mass standards (GE Healthcare Life Science, UK).

2.3.11 β-Lactamase Mass Characterisation

The molecular mass of the AmpC β-lactamase from *Enterobacter cloacae* sp. was determined according to Stachyra et al. (2010) in positive-ion mode by electrospray ionization mass spectrometry on a Q-TOF Voyager-DE PRO with Data Explorer processing software. Samples were prepared with sinapinic acid at 10 mg/mL in 50:50 ACN/H_2O + 0.1 % trifluoroacetic acid (TFA).

2.3.12 β-lactamase Induction Assay

To determine the influence of LY2183240 on β-lactamase induction, a colorimetric assay employing nitrocefin was used. Nitrocefin is a chromogenic cephalosporin that changes colour (yellow to red) on hydrolysis, providing a sensitive test for most β-lactamases (O'Callaghan et al., 1972) (See Figure 2.3).

Cultures of *Citrobacter freundii* 382010 growing exponentially were mixed with meropenem (10 times above the MIC to induce β-lactamase expression) (positive control) and LY2183240 regioisomers (0 - 420 µM) during a 1-hour period. Clavulanic acid was used as a standard inhibitor control. Subsequently, 20 µL of nitrocefin (100 µM, final concentration) were added to assess β-lactamase activity at 486 nm using an UV-visible spectrophotometer (Thermo-Scientific, UK).

β-Lactamase activity was indicated by a red colour change within 1-3 min (Figure 2.3). Reactions taking more than 10 minutes were not considered, as they may reflect the secondary β-lactamase activity of penicillin-binding proteins that form unstable acyl complexes (Livermore & Brown, 2001).

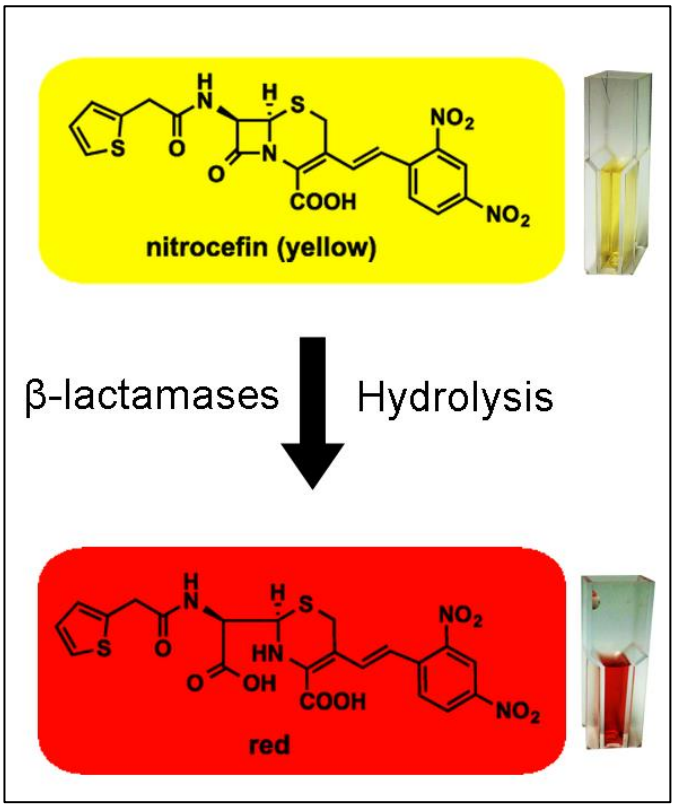


Figure 2.3. The reaction scheme for the hydrolysis of nitrocefin by β-lactamases. The cuvettes show the difference in the colour visually perceptible before (top, yellow) and after hydrolysis (bottom, red).

2.3.13 β-Lactamase Inhibition Studies

Class A and B β-lactamase activities were assessed spectrophotometrically by hydrolysis of the substrate nitrocefin according to O'Callaghan et al. (1972), with modifications. The assay mixture contained the β-lactamase (2.5 nM, TEM-1 or the growth medium supernatant of *K. pneumoniae* expressing NDM-1) and 100 μM of nitrocefin in a final volume of 1 mL of 50 mM phosphate buffer (PBS). β-Lactamase activity was monitored by measuring the absorbance at 486 nm for 3 min at 25°C. Both LY2183240 regioisomers were incorporated into the assay mixture separately and the β-lactamase activity measured as described above. Clavulanic acid was used as reference inhibitor for the class A enzyme.

2.3.13.1 Determination of IC_{50}

The inhibitor concentration that caused a 50 % reduction in enzyme activity (IC_{50}) was determined according to Bush et al. (1993), with modifications. Briefly, the activity data were obtained after 10 min of pre-incubation of 10 to 50 µL of the enzyme and LY2183240 regioisomers in different concentrations (0 – 420 µM) at 25°C. The enzymatic reaction started with the addition of the substrate nitrocefin at a fixed concentration of 100 µM in a volume of 1 mL. The data was determined graphically using GraphPad Prism version 7 software. Clavulanic acid, tazobactam and avibactam were used as standards.

2.3.13.2 Progressive Inhibition Determinations

The progressive inhibition determinations were performed according to Bush et al. (1993), with modifications. Briefly, the purified AmpC enzyme from *Enterobacter cloacae* (2.5 nM) and different concentrations of LY2183240 regioisomers were incubated together at 25°C in a total volume of 100 µL. A control containing PBS buffer was also prepared. At fixed time points, samples were promptly diluted (1:100) in reaction buffer containing 100 µM of nitrocefin. Hydrolysis rates were monitored over 3 to 5 minutes using a UV-visible spectrophotometer at 486 nm. All experiments were performed in triplicate. Tazobactam and avibactam were used as standards.

2.3.13.3 Kinetic Interactions Studies

For the kinetic studies, the inhibitory activity of the purified AmpC β-lactamase from *E. cloacae* sp. previously obtained was assessed using the chromogenic substrate nitrocefin as described above (O'Callaghan et al., 1972). Enzyme preparations were diluted in Phosphate-Buffered Saline (PBS), pH 7.0, in different sterile tubular universal flasks. A five-fold concentrated stock of nitrocefin was prepared in 30 % DMSO. Experiments employed a range of substrate concentrations (2.5 – 40 µM) and the enzyme AmpC (2.5 nM). The class A TEM-1 β-lactamase (0.25 µM) was used for method validation and comparison purposes. After incubating the enzyme at room temperature for 5 minutes in the presence of inhibitor, reactions were initiated

by the addition of nitrocefin in a final volume of 1 mL. Nitrocefin hydrolysis was monitored at 486 nm using a UV-visible spectrophotometer (Thermo Scientific, UK) controlled by the software package VisionPro (Thermo Scientific, UK). Reaction rates were linear from 0 – 3 minutes at room temperature (~ 25°C). All the main kinetic parameters were determined by non-linear regression analysis applying Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_m + [S]} = \frac{V_{max}}{1 + \frac{K_m}{[S]}} \tag{3}$$

This equation plots the reaction velocity (v) as a function of the substrate concentration [S] for an enzyme showing that the maximal velocity (V_{max}) is approached asymptotically. The Michaelis-Menten constant (K_m) is the substrate concentration yielding a velocity of $V_{max}/2$.

Followed by Lineweaver-Burk equation:

$$\frac{1}{V} = \frac{K_m + [S]}{V_{max}[S]} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \tag{4}$$

Equation K_{cat}:

$$V_{max} = K_{cat}\,[E_t] \tag{5}$$

Reported values for K_i were determined using a model for mixed inhibition. The mixed inhibition model is a general equation that includes competitive, uncompetitive and non-competitive inhibition as special cases (Copeland, 2000; Copeland, 2013):

$$v = \frac{\frac{V_{max}}{\left(1 + \frac{[I]}{\propto k_i}\right)}[S]}{[S] + K_m \left(\frac{\left(1 + \frac{[I]}{K_i}\right)}{\left(1 + \frac{[I]}{\propto K_i}\right)}\right)} \tag{6}$$

The model possesses one more parameter compared to the others, and this parameter indicates the potential mechanism of inhibition. Once the mechanism of inhibition was determined, the model of competitive inhibition was applied:

$$v = \frac{V_{max}\,[S]}{[S] + K_M\left(1 + \frac{[I]}{K_i}\right)} \tag{7}$$

All the data were conducted in triplicate and calculated using GraphPad Prism 7 software for fitting of data, statistical and error analysis.

2.3.14 *In Silico* Study

2.3.14.1 Molecular Modelling

Computer-based techniques for predicting the structure of ligand–protein complexes as well as docking algorithms can be used in both drug design and the elucidation of biochemical processes, including the understanding of the mode of action of a drug towards an enzyme target (Mcconkey et al., 2002).

Usually, the main purpose of molecular docking is estimating the ligand-protein complex structure using computational approaches. A docking can be achieved by three interconnected elements: identification of the binding site, a search algorithm to successfully sample the search space, e.g., the range of potential ligand positions and conformations on the protein surface, and a scoring function. Preferably, sampling algorithms should be able to replicate the experimental binding form and the scoring function should identify the most favourable complex or those with the highest affinity amongst all conformations provided (Meng et al., 2011; Mcconkey et al., 2002). The package software iGEMDOCK requires entry of the coordinates of the target protein atoms from the PDB (Protein Data Bank) format file and consecutively the atom coordinates of a ligand from the prepared ligand database. In this study, the ligands and the cephalosporinase were prepared and progressively used in the flexible docking mode for each ligand by using the genetic algorithm implemented in GEMDOCK (Yang & Chen, 2004; Barata et al., 2016). Its energy function comprises electrostatic, steric, and hydrogen-bonding potentials. The last two functions utilize a linear model, which is simple and distinguishes probable complexes promptly (Hsu et al., 2011).

2.3.14.2 Preparation of Proteins and Compounds

The structures of all ligands assessed were prepared with Avogadro 1.90.0. Protein crystal structures were obtained from the Protein Data Bank (PDB), http://www.rcsb.org/; β-lactamase from *Enterobacter cloacae* P99 is PDB entry 1xx2. The protein structure was verified and optimized with the Preparation Wizard (Prep Wiz) tool from Maestro 10.3. For validation purposes, two different standard β-lactamase inhibitors, tazobactam and avibactam, were also used as ligands in docking.

2.3.14.3 Water Molecules

Molecular docking accuracy and enrichment results can be affected by the treatment of explicit water molecules, as formerly revealed by some researchers (Wong & Lightstone, 2011; Corbeil & Moitessier, 2009; Verdonk et al., 2005; de Graaf et al., 2006; Myrianthopoulos et al., 2013). Nevertheless, the determination of which waters to preserve is challenging, mainly considering that the free energy of a water molecule is not precisely related to the crystallographic occupancy (Michel et al., 2009). Since the main purpose of this part of the study was to assess the performance of LY2183240 regioisomers towards class C β-lactamases and not to comprehensively investigate each possible treatment of water molecules, it was only considered whether or not to retain the water molecules in the β-lactamase structure for the hydrogen bond optimization and enzyme minimization stages. Possibly, with no water molecules, the hydrogen-bonding arrangements necessary for ligand binding could be disturbed or the β-lactamase could collapse. Conversely, in most cases, water molecules can raise several complications, such as the process of finding poses and calculating binding affinity (Wong & Lightstone, 2011). In addition, solvation is frequently neglected in docking scoring functions, making potential biased scoring results. In this sense, all water molecules were removed with Maestro 10.3 preceding molecular docking.

2.3.14.4 Protein-Ligand Molecular Docking

All the molecular dockings were performed using iGEMDOCK software (Hsu et al., 2011). This flexible and easy-to-use software adopts a genetic evolutionary method

and an empirical scoring function (Hsu et al., 2011; Yang & Chen, 2004; Barata et al., 2016). The scoring function is given as:

$$E_{tot} = E_{bind} + E_{pharma} + E_{ligpre}, \quad (8)$$

where E_{bind} accounts for inter- and intra-molecular energies and ligand is outside the search box, and fixed value is 10,000. The E_{pharma} term is the sum of all hot-spot atoms of the interaction between ligand and protein:

$$E_{pharma} = \sum_{i-1}^{lig} \sum_{j-1}^{hs} f(w_j, B_{ij}) F\left(r_{ij}^{B_{ij}}\right), \quad (9)$$

where w_j is the pharmacophore weight of the hot-spot atom j, $r_{ij}^{B_{ij}}$ is the distance between the atoms i and j with the interaction type B_{ij} forming by the pair-wise heavy atoms between ligands and proteins; B_{ij} is either a hydrogen bond or a steric state; *lig* is the number of heavy atoms in the ligand; and *hs* is the number of hot-spot atoms in the receptor. The value of f (w_j, B_{ij}) is w_j or *0*. f (w_j, B_{ij}) is w_j if the interaction type (B_{ij}) equals the type of hotspots found on the target receptor. The ligand preferences include electrostatic (i.e., the number of electrostatic atoms) and hydrophilic characteristic (i.e., the atom numbers of hydrogen donor and acceptor). The E_{ligpre} is a penalty value for a ligand, which is unable to satisfy the ligand preferences and is defined as

$$E_{ligpre} = WP_{elec} + WP_{hb} \quad (10)$$

where WP_{elec} and WP_{hb} are the penalties for the electrostatic and hydrophilic preferences, respectively (Hsu et al., 2011; Yang & Chen, 2004; Barata et al., 2016; Yang, 2004).

The docking used a customized protocol by setting a population size of 500, with 100 generations and 20 solutions. After the completion of the docking, post-docking analysis was performed to find the docking pose and its energy values. Confirmation of the scoring process was performed by the software package VEGA ZZ version 3.0.5.12 (Pedretti et al., 2004).

All the results were processed using the software packages Maestro 10.3, Pymol 1.3 (Schrödinger, LLC, New York, NY, USA) and Discovery Studio Visualizer 4.0 (Biovia, San Diego, CA, USA).

2.3.15 Characterization of the Enzyme-Inhibitor Complex

The purified AmpC enzyme from *E. cloacae* (40 μM) was incubated for 15 min at 37°C in the presence and absence of 4 mM avibactam, 1,5-LY2183240 or 2,5-LY2183240, dialyzed at 4°C and analysed by MALDI (Matrix Assisted Laser Desorption/Ionization) –TOF (Time of Flying) mass spectrometry (MS) (Stachyra et al., 2010).

For all the analyses Voyager-DE PRO with Data Explorer processing software was used. Samples were prepared for MALDI-TOF with sinapinic acid at 10 mg/mL in 50:50 ACN/H_2O + 0.1 % trifluoroacetic acid (TFA). Table 8 summarizes the details of the parameters used.

Table 6. MALDI-TOF parameters.

Mode of operation:	Linear
Polarity:	Positive
Extraction mode:	Delayed
Accelerating voltage:	25000V
Grid Voltage	92 %
Guide wire:	0.3 %
Extraction delay time:	750-1500 *n*sec

2.3.16 Crystallographic Study

The crystallization techniques were conducted according to those described by Professor Knox's research group (Lobkovsky et al., 1993; Lobkovsky et al., 1994; Crichlow et al., 1999).

2.3.16.1 Crystallisation

Crystals of the 39,134 Da β-lactamase were grown at room temperature by the sitting-drop vapour diffusion method using 1:1 or 3:2 ratios of protein to reservoir solutions. Protein at 6.2 mg/mL was diluted with reservoir solution containing 20 % PEG 8,000 (Sigma-Aldrich, UK) and 0.1-M KH_2PO_4 at pH 5.0.

2.3.16.2 Preliminary X-ray Data Collection

Crystallographic data were collected at the Diamond LightSource on beam line Io4-1, at a wavelength of 0.9785 Å, and 100 K. Processing and data reduction were carried out on site using Xia2, an automated data processing tool within CCP4 (Wei et al., 2012).

3 CHAPTER 3

Mini-Screening of “legal high” natural products and synthetic compound LY2183240 for antibacterial and enzyme inhibition effects

3.1 Introduction

The discovery and clinical use of antimicrobial agents in the 1950s have unquestionably given one of the greatest benefits to humankind (Bérdy, 2012).

Over the following years, the average lifespan of the population drastically increased, some infectious diseases almost disappeared, and several viral diseases became controllable. In addition, antibiotics were established to be useful in the treatment of bacterial, fungal and protozoan infections and some physiological diseases. These agents were also used in veterinary applications, as plant-protecting agents and as feed additives. As a result, the misuse and overuse of these compounds has led to a global antimicrobial resistance crisis, representing a significant public health menace and challenge. There is a current need to develop new antimicrobial agents affective against antibiotic-resistant organisms.

Plants are rich in an extensive diversity of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been revealed to have antimicrobial properties *in vitro* (Cowan, 1999). Indeed, most existing antibiotics are derived from natural products, being a rational approach to discover potential novel compounds with antimicrobial and resistance-modifying properties (Brown & Wright, 2016).

This chapter describes a mini-screening of unusual sources of natural products (‘legal highs’) and the synthetic compound LY2183240 for antibacterial effects.

It is noteworthy that the data described in this chapter have been developed progressively and insofar as the results were obtained. In other words, the decisions of the next step in the study were a consequence of the result from the previous experiment.

3.2 Objectives

Antimicrobial resistance is a worldwide reality and this is a trend that will increase in scale rather than decrease. Therefore, development of novel strategies and compounds that tackle this issue are important and vital. It is well known that natural products from plant sources produce antibacterial metabolites. Many studies from the Research Department of Pharmaceutical and Biological Chemistry of UCL School of Pharmacy have demonstrated interesting antimicrobial activities from different species, including 'legal highs'. Furthermore, some psychoactive compounds from plants exhibit targets that are similar to those present in prokaryotic cells.

The main objective of this chapter is to investigate the potential antimicrobial activity of crude extracts of different species of plants considered as legal highs and the synthetic compound LY2183240.

The specific aims of this section of the study were as follows:

- To solvent extract the plant materials of *Trichocereus peruvianus, Trichocereus pachanoi, Mitragyna speciosa, Argyneia nervosa, Banisteriopsis caapi, Salvia divinorum, Amanita muscaria, Morning glory* and *Leonotis leonurus* with *n*-hexane, dichloromethane, chloroform and methanol in order to obtain standardized extracts to test;
- To assess the antimicrobial activity by determining the minimum inhibitory concentrations (MIC) of the extracts and LY2183240 against a broad range of bacteria;
- To determine the minimum bactericidal concentration (MBC) of compound LY2183240 against a broad range of bacteria;
- To evaluate the ability of LY2183240 to inhibit prokaryotic hydrolases;

3.3 Results and Discussion

3.4 Crude Extracts and Antimicrobial Activity

The antimicrobial effects of several plants species investigated in this study have been well documented over the years with evidence supporting the efficacy of many natural products as antibacterial agents (Cowan, 1999). In general, one of the approaches to evaluate the antimicrobial activity of natural products is to grade activities such that extracts which display an MIC less than 100 µg/mL, are regarded as good; from 100 to 500 µg/mL, the activity is considered moderate; from 500 to 1000 µg/mL the activity is weak; and over 1000 µg/mL the extracts are considered inactive. In this study, the antimicrobial potential of several plant species considered as 'legal highs' were investigated against different bacteria.

The extraction methods applied in this work yielded twenty-eight extracts from nine species, after employing *n*-hexane, chloroform, dichloromethane and methanol as solvents.

Figure 3.1. Extract preparations of the main species used in the mini-screening for antimicrobial activity. Photo: P. E. De Resende.

The antimicrobial activities of all extracts were assessed by the broth microdilution technique against four Gram-positive and four Gram-negative bacteria. None of the extracts showed potent antimicrobial activity with MIC values exceeding 1028 µg/mL for all organisms tested. However, these results do not fully corroborate with previous studies; in a screening study using medicinal plants from Northern Peru, the antimicrobial activity of Ayahuasca (*Banisteriopsis caapi*) extract against *E. coli*

gave an MIC of 62.5 µg/ml (Bussmann et al., 2010) . However, consistent with this study the extract had no activity against Gram-positive species.

Regarding *Argyreia nervosa*, Joshi et al. (2013) demonstrated inhibitory activity against various species of *Bacillus* (MIC 59.7 to 373.2 µg/mL). Another study showed antimicrobial activity for extracts of *Salvia*, however, they did not include the specific species *Salvia divinorum* used in this study (Khalil et al., 2005). Furthermore, it has been reported that extracts from *Mitragyna speciosa* had some antibacterial effect against *B. subtillis*, however the study reported a high MIC range of 3.12 to 6.25 mg/mL, which may well represent weak or no activity (Parthasarathy et al., 2009).

The dissimilar results found in this work when compared with other studies utilizing the same species may be due to different extraction methods employed, source of plant material, and different bacterial strains used. Since plants were purchased online, doubt concerning their authenticity may exist. The possibility of contaminants interfering with the purity of the extraction or the action of the phytochemicals against the microbes may have contributed to the unexpected outcomes.

Furthermore, the 'legal high' plants used in this study have a complex natural chemistry and the few studies that have been conducted so far are considered dated (Arunotayanun & Gibbons, 2012). In addition, for obvious reasons, most of the studies were generally focused on their effects on the central nervous system, rather than the antimicrobial activities of these species.

3.5 Antibiotic Potentiation Activity of Plant Extracts

In order to evaluate the potential antibiotic resistance modifying activity of the extracts prepared, the MIC of amoxicillin were determined in the presence or absence of the test samples using the broth microdilution technique. All extracts were used at the concentration of 512 µg/mL and the standard reference amoxicillin at a range of 0 to 128 µg/mL. The main results are summarised in Table 4.

None of the extracts showed any positive modification in the activity of amoxicillin against the microorganisms tested. In fact, some of the extracts of *T. pachanoi* and *S. divinorum* increased the amoxicillin MIC up to 256-fold, showing a negative influence towards the antibiotic activity.

Potential inhibitors of drug resistance mechanisms may have great value because they could make resistant bacteria sensitive to drugs (Horiuchi et al., 2007). Nevertheless, due to these findings, the antimicrobial and resistance-modifying activities of the extracts were not studied further and the project focused entirely on the properties of LY2183240 regioisomers.

Table 7. MIC of amoxicillin in the presence and absence of different plant extracts against amoxicillin-susceptible and -resistant bacteria.

Antibiotic	**Extract**		***S. aureus* 12981**	***S. aureus* 13373**	***E. coli* 10418**	***E. coli* G69**
AMX			**0.25**	**8**	**4**	**>128**
	T. peruvianus	HEX	1	16	16	>128
		ETA	1	32	16	>128
		MET	0.5	8	16	>128
	T. pachanoi	HEX	4	8	4	>128
		ETA	64	8	8	>128
		MET	8	8	8	>128
	M. speciosa	HEX	2	16	1	>128
		ETA	4	8	4	>128
		MET	0.5	8	16	>128
	A. nervosa	HEX	1	16	8	>128
		ETA	4	32	8	>128
		MET	2	8	4	>128
	B. caapi	HEX	2	32	8	>128
		ETA	8	64	16	>128
		MET	16	64	16	>128
	S. divinorum	HEX	4	4	4	>128
		ETA	64	8	8	>128
		MET	8	8	8	>128
	A. muscaria	HEX	1	16	16	>128
		ETA	2	32	16	>128
		MET	1	4	8	>128
	L. leonurus	HEX	2	16	8	>128
		ETA	1	16	8	>128
		MET	0.5	32	16	>128

AMX, amoxicillin; HEX, *n*-hexane; ETA, ethyl acetate; MET, methanol.

3.6 Characterisation of LY2183240

3.6.1 Thin Layer Chromatography

Although there is a tendency towards utilizing HPLC in analyzing chemical mixtures, thin-layer chromatography (TLC) is still a very popular and a commonly used analytical method in industry and academia (Ferenczi-Fodor et al., 2011) and was particularly effective in this case. It is noteworthy that since the sample LY2183240 was purchased online, there was a lack of information about the ingredients, chemistries, biological activities, pharmacology and toxicology provided. Hence, the need to establish whether the 'compound' purchased was pure.

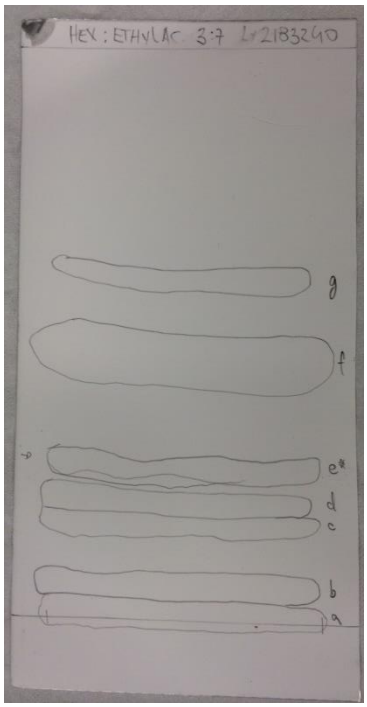


Figure 3.2. A TLC plate of LY2183240 separated with a mobile phase of hexane-ethyl acetate 3:7. It is possible to see at least six bands at 240 nm and 1 at 360 nm (UV light).

The isolation of seven different compounds was evident upon TLC analysis, indicating that it is a mixture containing potential impurities (Figure 3.2). The separated compounds are shown encircled in pencil after viewing at 240 nm. It is notable that band "e" was visualized at 360 nm. The sixth band, named "f", is the major compound present in this mixture and may correspond to LY2183240.

3.6.2 High Performance Liquid Chromatography (HPLC)

Subsequent to the TLC test, characterisation of LY2183240 was performed by HPLC analysis. It was possible to visualize three distinctive peaks at retention times of 6.16, 6.48 and 6.75 minutes (Figure 3.3). The peaks at 6.48 and 6.75 minutes demonstrated to be 50 % and 39 % of the sample, respectively. The first peak, conversely, was 10 % of the tested sample.

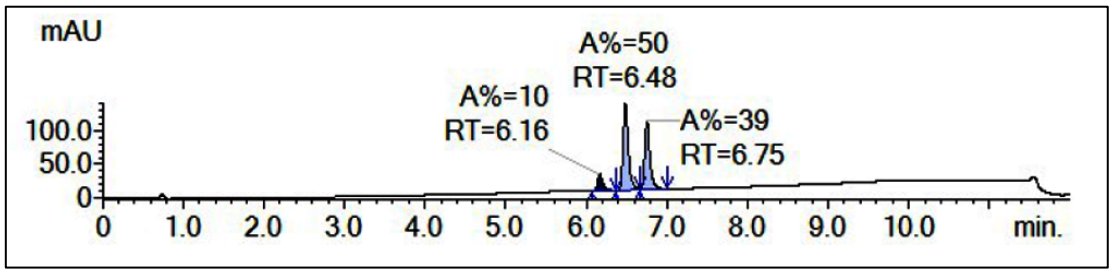


Figure 3.3. HPLC chromatogram of LY2183240.

Based on this analysis, the sample was not pure, consisting of at least 3 different compounds, 2 major compounds and one potential impurity.

3.6.3 Spectroscopic Analyses

It is generally accepted that a single analytical technique will not provide sufficient determination of the compound investigated and therefore multiple analyses from different technologies are necessary for a comprehensive view (Summer et al., 2003; Young et al., 2004). Nevertheless, it is acceptable to employ a single wide-spectrum chemical analytical technique, which is rapid, reproducible, and stable in time. NMR and mass spectrometry are techniques that meet those requirements.

Figure 3.4 depicts the proton one-dimension NMR spectrum of the LY2183240 mixture. Similar patterns were observed by visual inspections of ^{1}H-NMR spectra of a 99%-pure sample of LY2183240, purchased from a different source. The signals of LY2183240 (^{1}H-NMR – 400 MHz, Methanol) 7.6-7.5 (m, 5H), 7.4-7.3 (m, 4H), 4.5 (s, 1H), 4.4-4.3 (s, 6H) correlated with previous data (Alexander & Cravatt, 2006). However, the signals in the lower field spectrum probably correspond to impurities or other compounds present in the mixture. Although a more complete analysis of NMR and other techniques are needed to comprehend better the LY2183240 mixture, it was possible to observe the presence of LY2183240 in the sample analysed.

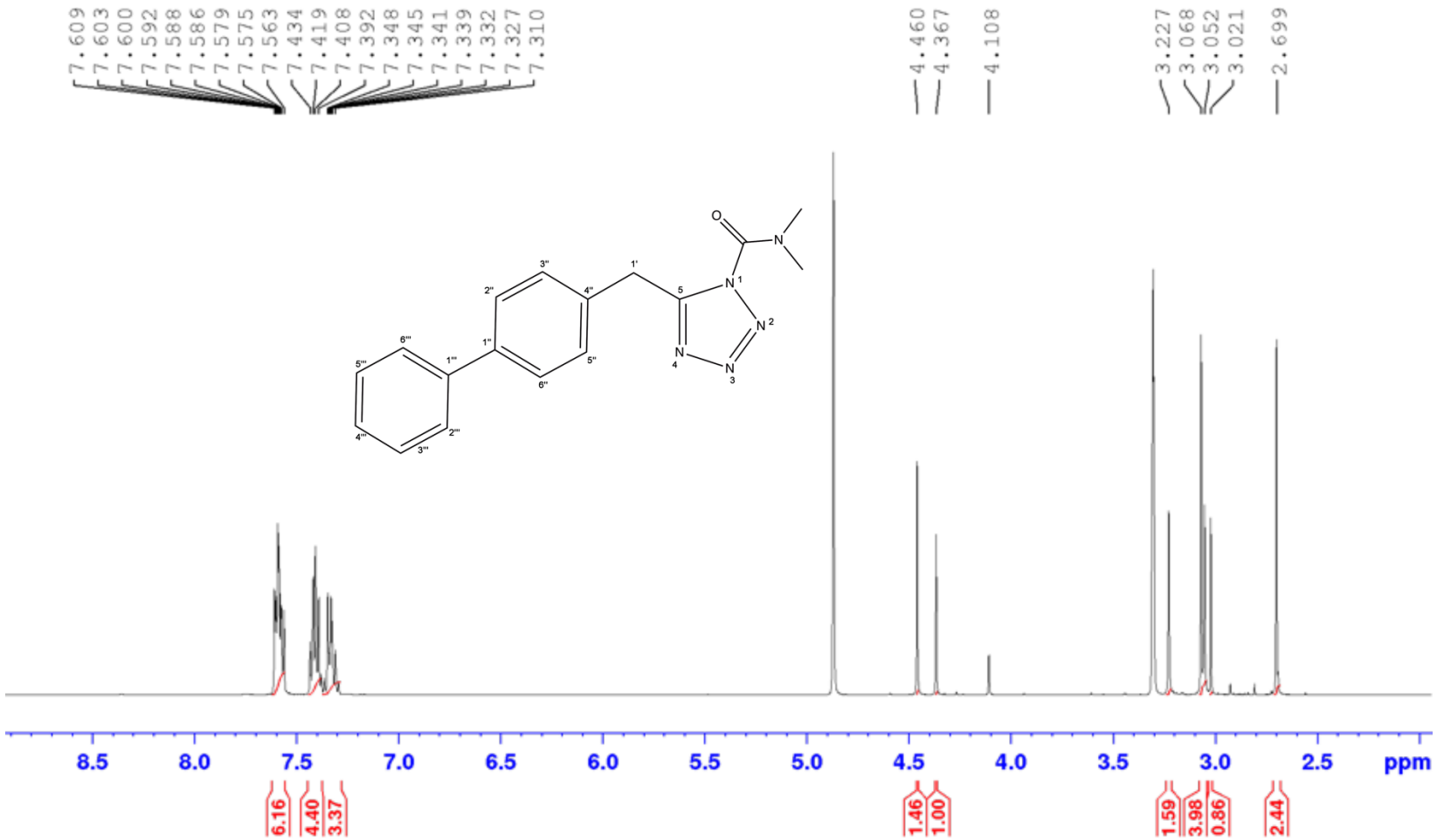


Figure 3.4. ^{1}H NMR spectrum for LY2183240 mixture, recorded in Methanol-d_4, 500MHz. The methanol peak is set at 3.31 and 4.87 ppm.

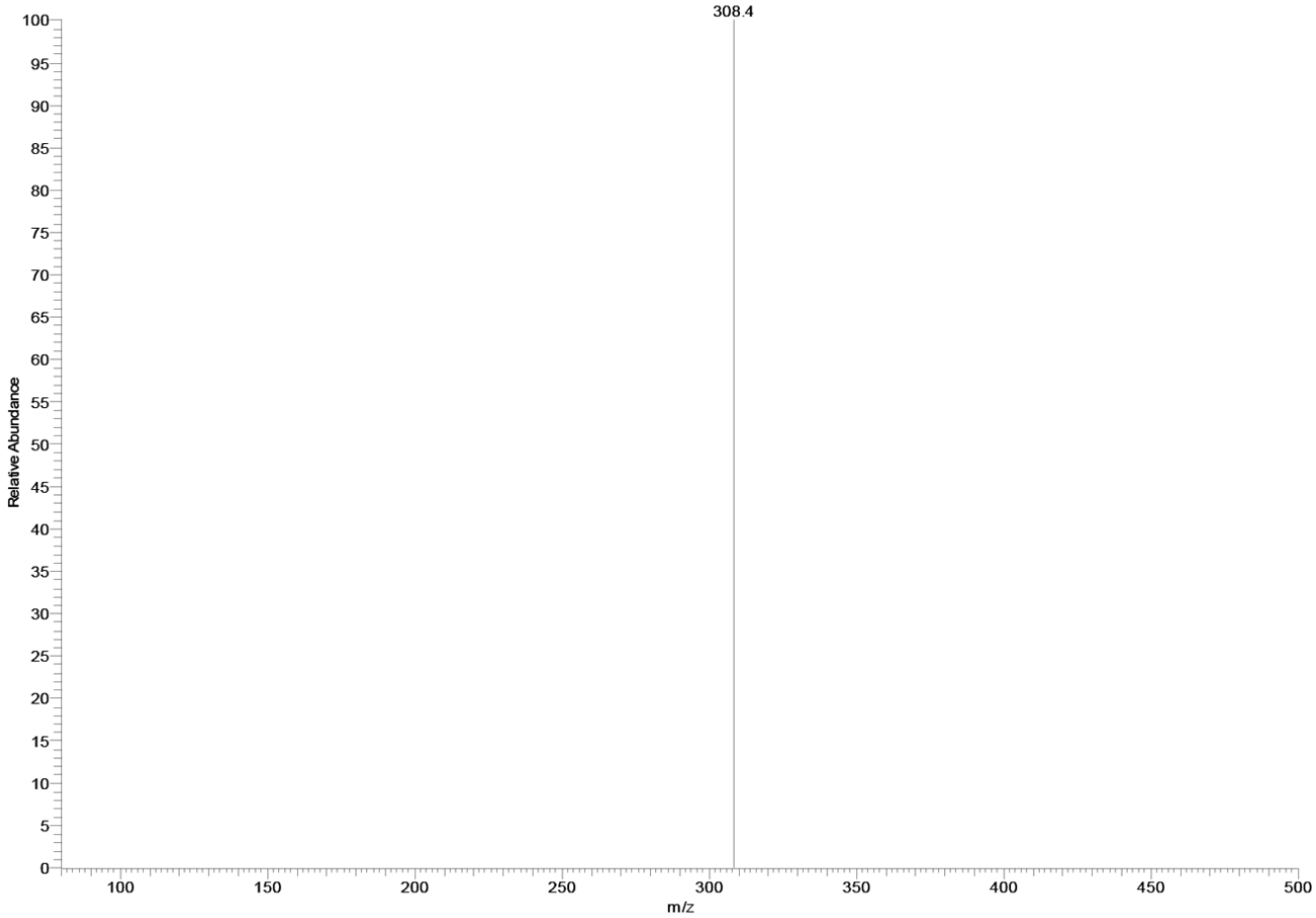


Figure 3.5. ESI-MS/MS spectrum for LY2183240.

The mass spectrometry analysis showed a single peak at 308 *m/z*, which matches with the molecular weight of LY2183240. This finding together with the HPLC and NMR analyses confirm the presence of LY2183240 in the sample, although it revealed the sample to be not a pure compound, but a mixture of at least two major compounds, potentially two regioisomers of LY2183240 since the same molecular weight was observed.

All the biological assessment of this present chapter was performed using this sample, nevertheless Chapter 4 will explore more the antimicrobial activity of the compounds present, separately.

3.7 Biological Evaluation

3.7.1 Antibacterial Activity of the LY2183240 Mixture

Table 8 shows the antimicrobial activity of LY2183240 mixture against a wide range of microorganisms. The mixture showed activity against methicillin-susceptible *S. aureus*, *B. subtilis*, and to a lesser extent methicillin-resistant *S. aureus* 13373. However, it had no activity against any of the Gram-negative organisms evaluated in this study. The results suggested that LY2183240 had specific activity against certain Gram-positive bacteria; for example, no effect on *E. faecalis* growth was observed for the concentration range investigated.

Table 8. Minimum inhibitory concentration of LY2183240 mixture determined by a microdilution assay.

Microorganisms	**MIC (µg/ml)**	**MBC (µg/ml)**
Escherichia coli NCTC 10418	>128	>1,024
Klebsiella pneumoniae 17	>128	>1,024
Pseudomonas aeruginosa 10663	>128	>1,024
Stenotrophomonas ssp.	>128	>1,024
Staphyloccocus aureus 12981	16	>1,024
Staphyloccocus aureus 13373	64	>1,024
Enterococcus faecalis 12697	>128	>1,024
Bacillus subtilis 13	16	>1,024

Gram-positive bacteria are deficient in many of the complex permeation and export pathways found in Gram-negative organisms, making them generally more susceptible to antimicrobial therapy (Coleman 2004). However, this class of microbes are responsible for a wide range of diseases and Gram-positive bacterial-related infections impose a major burden on patients and the health system globally (Rybak 2001). Additionally, the spread of new multi-drug resistant variants of *S. aureus* in the hospital and community is causing major concern (Daum & Seal, 2001). The fact that compound LY2183240 demonstrated selective activity against Gram-positive bacteria, particularly *S. aureus,* makes it a subject of further exploration, especially in terms of mechanism of action. Moreover, results from the MBC assay demonstrated the presence of bacterial growth after 24h-incubation. This data suggests that LY2183240 exhibited bacteriostatic activity.

From the results obtained and in order to understand the specific mode of action of LY2183240 against *S. aureus* and *B. subtilis,* a range of different experiments and assays were conducted.

3.7.2 Bioautographic Agar Overlay Assay

Bioautography is a technique that combines TLC with both biological and chemical detection methods. Many studies have been done on the screening of extracts from different sources, mostly from plants, for antimicrobial and antifungal activity utilizing this method (Horváth et al., 2010; Balouiri et al., 2016).

In the case of LY2183240, the presence of two main bands were observed on the TLC plate and confirmed by HPLC analysis. The main goal of the bioautography technique was to verify if both compounds present in the mixture exhibited antimicrobial activity.

Figure 3.6 illustrates the autobiogram of the TLC of LY2183240 mixture against a susceptible strain of *S. aureus* (NCTC 12981). It was observed that the band at the top of the TLC plate (see Figure 3.2) gave rise to a zone of inhibition against the Gram-positive strain tested. By contrast, the band in the bottom showed no effect at all, suggesting that only one compound present in the mixture possess antibacterial activity. It is noteworthy that the other bands present in the sample were considered impurities and were excluded in this assay. Nonetheless, all the bands were assessed in a paper diffusion assay and further discussed in this chapter.

The bioautographic method can be a useful tool both for the analytical determination of the main compounds within complex mixtures and for characterization of their biological properties (Choma & Jesionek, 2015). The discovery of clavulanic acid, for instance, was carried out using this technique, revealing its benefit, especially in preliminary studies (Reading et al., 1977). In this study, the bioautography was successfully applied demonstrating the potential component responsible for the antimicrobial activity in the LY2183240 mixture. The next section, hence, moves on to explore more this intriguing effect.

Figure 3.6. Bioautogram of the two main bands of LY218340 mixture against *S. aureus* 12981.

3.7.3 Paper Disc Diffusion Assay

Since the discovery of antimicrobials, the issue of drug resistance among pathogens became evident. The disk diffusion test, based on radial diffusion of antimicrobial agents from paper disks, has been playing a significant role through the years, being

well suited for testing pathogens isolated in both clinical and microbiology research environments (Kronvall et al., 2011).

In order to test all compounds present in the mixture, the seven bands present in the TLC plate (See Figure 3.2) were extracted from the silica and subjected to the paper disc diffusion assay. It was observed that only band "f", the major compound in the mixture, exhibited antibacterial activity against the different strains of *S. aureus* evaluated (Figure 3.7). This finding corroborates the previous bioautogram obtained from section 3.7.2 above. This assay showed that LY2183240 has potential antibacterial activity against *S. aureus* 12981 and *S. aureus* 13373 (MRSA), but further research is necessary to confirm this effect.

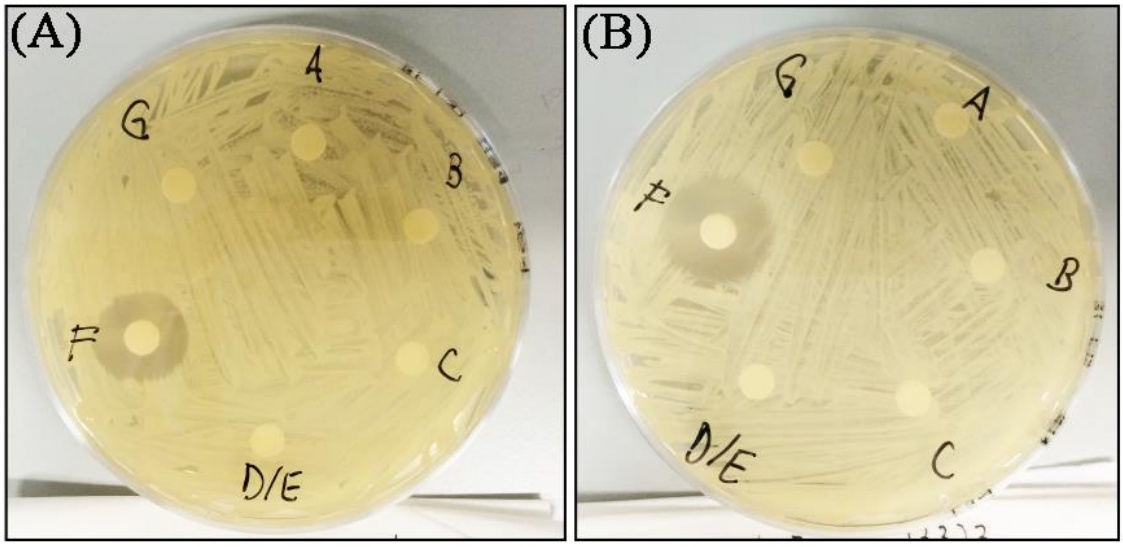


Figure 3.7. Antibacterial activity of the bands found in LY2183240 mixture assessed by paper diffusion assay using two different strains of *S. aureus*: (A) 12981 (MSSA) and (B) 13373 (MRSA).

3.7.4 Potential Anti-Conjugative Activity of LY2183240

Apart from chromosomal DNA, bacteria can carry one or more extra-chromosomal, double stranded, circular or linear DNA molecules, called plasmids. Plasmids are capable of self-replication and they maintain a substantial diversity of genes, such as those that ensure stable inheritance during cell division, as well as genes that confer antibiotic resistance and virulence determinants (Bennett, 2008; Aminov, 2009). They do not present essential genes for growth or development of the cell; nonetheless they transport genetic information that may be valuable to permit survival in specific environmental situations, like the presence of a potentially toxic

antibiotic. Moreover, they evolve as an important part of the bacterial genome, providing resistance genes that can be easily exchanged among bacteria of different origin and source by conjugation (Carattoli et al., 2001).

In conjugation, plasmids are transferred through an external filamentous structure called sex *pili*, especially in Gram-negative bacteria and, less frequently, in gram-positive bacteria; both donor and recipient end up with a copy of the plasmid. The gene for β-lactamase production in resistant *Haemophilus* strains, for example, is on a conjugative plasmid and is believed to have been transferred from this species to gonococci, resulting in high-level resistance that is already became a major problem worldwide (McManus, 1997; Tenover 2006; Bennett, 2008).

Although compounds based on anti-plasmid approaches have not yet advanced to clinical trials, the well-documented occurrence of plasmids within the most problematic drug-resistant bacteria makes the targeting of plasmid-encoded elements an intriguing antibacterial option (Williams & Hergenrother, 2008).

Since plasmid conjugation is vastly relevant for antimicrobial resistance studies and considering that it is a topic and research subject within our research group, the potential antiplasmid activity of LY2183240 was assessed.

The inhibitory activity of LY2183240 against bacterial conjugation was evaluated in a conjugal transfer of PKM101, TP114, pUB307 and R7K plasmids, using a broth mating method. The transfer frequency was calculated as the number of transconjugant colonies to the number of the donor colonies, expressed as CFU/mL. The results are illustrated in Figure 3.8 as the percentage of transfer frequency compared to the activity of the control.

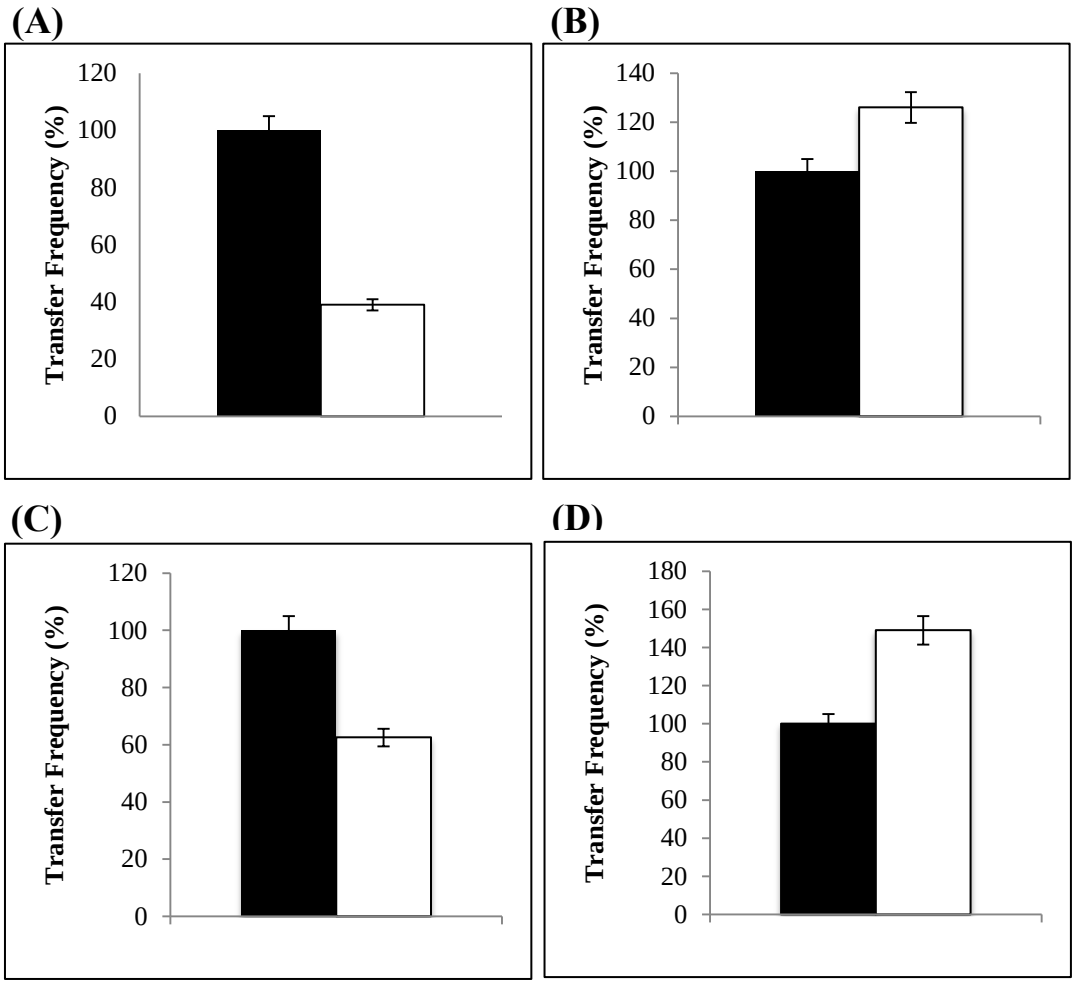


Figure 3.8 The percentage of transfer frequency using the plasmids: pKM101 **(A)**, TP114 **(B)**, pUB307 **(C)** and R7K **(D)**. Control (black bar), in the presence of LY2183240 (white bar).

In regards to pKM101 plasmid transfer, LY2183240 showed significant inhibitory activity of 61% ($p < 0.05$) (Figure 3.8 A). Similarly, LY2183240 exhibited a moderate inhibitory activity (47.5%) in the transfer of the plasmid pUB307 (Figure 6.7 C), although not statistically significant ($p > 0.05$). In contrast, with the plasmids TP114 and R7K, LY2183240 showed a marked increase of the conjugal transfer, with a transfer frequency percentage of 126 % and 149 %, respectively (Figures 3.8 B and D).

Research towards inhibitors of plasmid-mediated resistance is a relatively new and unexplored field. Molnár and coworkers (1992) evaluated the antiplasmid activity of promethazine and they demonstrated that this synthetic compound cured antibiotic resistance and lactose fermentation in *E. coli*. It was also shown that different plasmids of *E. coli* were eliminated with varying frequency. Moreover, the molecule was interfered with tumour-promoting ability of *Agrobacterium tumefaciens* and

nodule formation of *Rhizobium meliloti*. The authors ascribed the antiplasmid activity of the promethazine to the increased membrane permeability suggesting and inhibition of DNA gyrase and complex formation with the supercoiled form of plasmid DNA that leads to the cessation of plasmid replication in the bacterial cells. In addition, *in vivo* plasmid curing was demonstrated at a low frequency (Molnár et al., 1992). In a more recent work, Molnár et al. (2003) showed that the plasmid elimination efficiency of phenothiazine is markedly enhanced when a second species of bacteria is present, indicating that the mechanism of plasmid elimination is complex. Regarding natural products, the first report was described by Lakshmi et al. (1987), where plumbagin, a plant derived compound, cured R-plasmids in *E. coli*. In a study using *Plumbago zeylanica* extracts, it was observed an effect on R-plasmid harboring *E. coli* (Beg & Ahmad, 2000). Antiplasmid activity of essential oils was studied by Schelz et al. (2006) (Schelz, Molnar et al. 2006) and more recent, Shriram et al. (2008) (Shriram, Jahagirdar et al. 2008) have identified 8-epidiosbulbin E acetate as a potential plasmid-inhibitor agent from *Dioscorea bulbifera* L. against multi-drug resistant bacteria.

As far as we know, there have not been any previous studies on the effect of LY2183240 on the bacterial conjugation mechanism and this is the first report of plasmid transfer inhibition activity by this compound. Nevertheless, further investigation must be conducted in order to explain these observations.

3.7.5 LY2183240 May Interact With Cell Division Proteins

The process of cell growth and division involves the formation and assembly of new cell wall material along the length of the cell and at the division septum (Hayhurst et al., 2008). These processes are regulated by mechanisms controlling the expression and localization of the peptidoglycan biosynthetic machinery (Biller et al., 2011). Cefotaxime is a third-generation class of cephalosporin and as a β-lactam agent inhibits bacterial cell wall synthesis by binding to one or more of the PBPs. A low concentration of cefotaxime (0.01 to 1 μg/mL) is known to induce cell elongation of *Salmonella typhimurium* (Gale et al., 1981). Characterisation of PBPs led to the conclusion that cefotaxime binds selectively to PBP 2B, (M. Bamberger et al., 2012), a high-molecular-weight PBP. Several studies have provided conclusive evidence

that this protein is involved in cell division and mutant *B. subtilis* strains lacking sufficient amount of this protein fail to separate and form filamentous structures (Daniel et al., 2000).

In this study, an assessment of the effect of LY2183240 on the morphology of *B. subtilis* cells was used to verify if the compound affected cell wall synthesis or cell division and cefotaxime was used as a positive control in these experiments.

Figure 3.9 shows the results of cefotaxime (0.01 µg/mL) compared to that of LY2183240 (1.6 µg/mL) on the morphology of *B. subtilis* and *S. aureus* 12981. The inserts are representative of the change in cell morphology after 90 minutes of exposure to the drug. Both compounds had an effect on the morphology of the microorganisms tested. It is evident that the effect of cefotaxime is more prominent compared to the effect of LY2183240 on the cells, especially on *S. aureus*. The formation of larger grape like clusters can be seen when *S. aureus* cells had been treated with cefotaxime. In the presence of LY2183240, however, there was no obvious difference in the morphology or the arrangements of the spherical cells. The morphological response of LY2183240 on the cell shape of *B. subtilis* is very similar to that produced by cefotaxime on the rod shape bacilli. Filamentation and elongation of the cells can be seen although a larger proportion of cells seem to have been affected by the inhibitory action of cefotaxime than that of LY2183240, suggesting that the third generation cephalosporin is a more potent inhibitor of cell division.

Since cells treated with LY21803240 were similar in form to those treated with cefotaxime, this could indicate that LY2183240 is also involved in inhibiting enzymes responsible for cell separation.

Little consideration will be given to the morphological effects of *S. aureus*, since evidence of increase in cluster formation was not conclusive. Given that wild type *S. aureus* exists in clusters it seemed that the arrangements detected with the microscope were merely the protective formations usually assembled in the microbe species to provide the physical form of a biofilm barrier against hostile environments (Haaber et al., 2012).

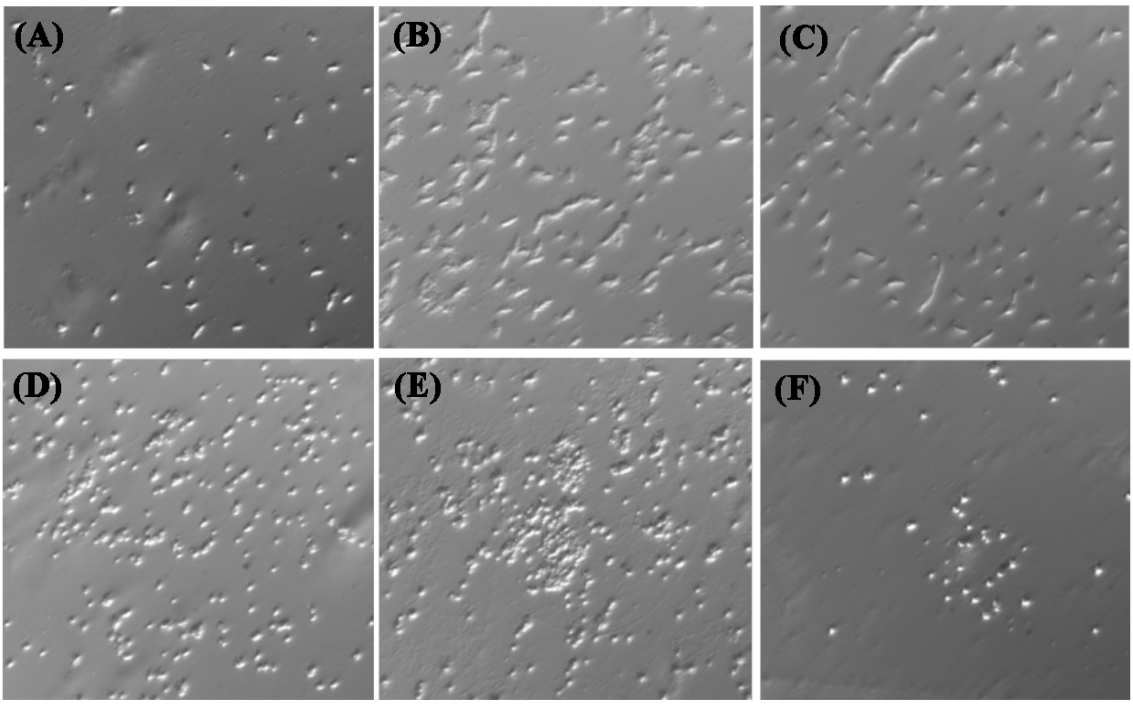


Figure 3.9. Morphological detail recorded with Zen Pro 2012 image software. Elongation of *B. subtilis* rod-shaped cells without any treatment **(A)**, after treatment with cefotaxime (0.01 µg/mL) **(B)**, and LY2183240 (1.6 µg/mL) **(C)**. Relative to the *S. aureus* 12981 control **(D)**, treatment with cefotaxime (0.01 µg/mL) **(E)** shows an increase cluster size and number; however the morphological effects of LY2183240 (1.6 µg/ml) on the spherical cells demonstrated no change **(F)**. All six microscopic photos were taken after a 90 min-interval of drug exposure. All images were recorded under the same magnification (x 100) phase contrast.

3.7.6 Modulation of Penicillin-Induced Cell Lysis

A few years ago, a report that describes LY2183240 as a highly potent small-molecule inhibitor of anandamide transport has motivated more interest in elucidating the protein(s) responsible for this process (Moore et al., 2005). In a more recent work, it has been demonstrated that LY2183240 is an extremely potent inhibitor of fatty acid amide hydrolase (FAAH), both *in vitro* and *in vivo*. This compound inactivates FAAH by covalently binding to the enzyme's serine nucleophile (Alexander & Cravatt, 2006). In addition, functional proteomic screens recognized numerous additional serine hydrolases that were also inhibited by LY2183240, suggesting that this compound has a promiscuous activity against this large and diverse enzyme class. However, none of these former studies reported inhibition of serine hydrolases present in prokaryotic cells, inspiring interest in investigating the action of LY2183240 on bacterial peptidoglycan hydrolases. In this

context, the main objective of this assay was to determine whether this compound has an effect on inhibiting endogenous *M. lysodeikticus* (ATCC 4698) cell hydrolases, counteracting therefore the action of penicillin G in lysing the cell.

LY2183240 exhibited a concentration-dependent inhibition of lysis of *M. lysodeikticus* cells. Figure 3.10 illustrates a decrease in the percentage of initial absorption according with time and LY2183240 concentration. For instance, at 120 minutes, a 21 % decrease in absorbance was observed ($p < 0.05$) in the presence of LY2183240 (128 µg/mL). In comparison, within the same timeframe, with the compound at 64, 32 and 0.125 µg/mL, a reduction in absorbance of 43, 58 and 69 % ($p < 0.05$) was noted, respectively.

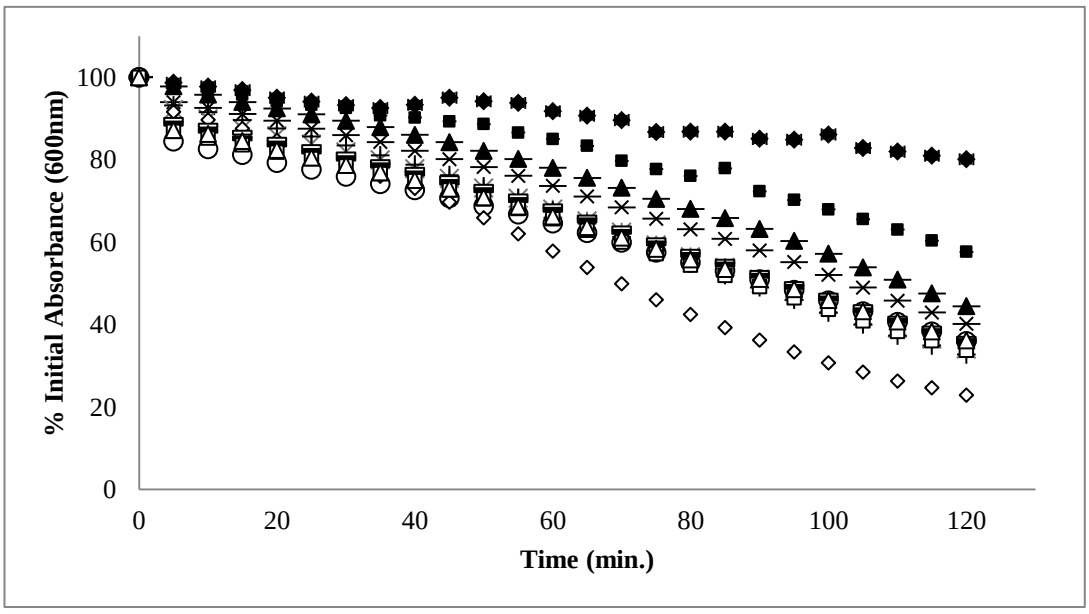


Figure 3.10. The effect of penicillin G in combination with LY2183240 on *Micrococcus lysodeikticus*. The graph illustrates penicillin G (200 µg/mL) in combination with LY2183240 in a concentration range of (◆, 128 µg/mL; ■ 64 µg/mL; ▲ 32 µg/mL; × 16 µg/mL; ● 8 µg/mL; ○ 4 µg/mL; + 2 µg/mL; ◇ 1 µg/mL; = 0.5 µg/mL; – 0.250 µg/mL; □ 0.125 µg/mL; △ 0 µg/mL). Changes in OD_{600} were measured at 5-minute intervals for 120 minutes. Data are representative of experiments preformed in triplicate.

β-Lactams are a broad class of antibiotics encompassing cephalosporin and penicillin derivatives, both of which were investigated in this study for comparison purposes against LY2183240. This class of agents binds covalently to penicillin-binding-proteins (PBPs) with different affinities dependent upon the individual drug. In general, β-lactams target the bacterial cell wall formation, specifically by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls (Kong et al., 2010).

Penicillin G, like other β-lactams, acts on actively growing cells to inhibit the terminal reaction of cell wall biosynthesis. This reaction is termed transpeptidation and involves the formation of a complex with transpeptidase and *D*-alanyl-*D*-alanine dipeptide.

Penicillin competes with *D*-alanyl-*D*-alanine for the active site of this PBP to form an inactive complex. As a result of its action the usually rigid peptidoglycan layer loses its integrity and the cell becomes unable to synthesis a typical matrix that can withstand the increase in internal osmotic pressure, ultimately leading to cell lysis. This would manifest itself as a decrease in turbidity as the number of intact cells decrease (Blumberg & Strominger 1974).

In addition, penicillin also lyses Gram-positive bacteria by activation of autolytic enzymes, the activity is triggered by release of an inhibitor from the bacterial cell wall (Tomasz, 1974; Fontana et al., 1977; Kiriyama et al., 1987).

The absorption measurements, obtained in the assay in the presence of penicillin G indicated a decrease in cell lysis with increased LY2183240 concentration. This is depicted in Figure 3.10 where the percentage decrease in turbidity is significantly lower in the presence of LY2183240. The data suggests that LY2183240 inhibits enzymes that would otherwise amplify the effect of penicillin G by causing cell lysis. This theory points to the inhibition of peptidoglycan hydrolases responsible for cleaving the muropeptides from the middle of the division septum to allow for the separation of the cells.

Multiple PBPs are found in microbes, of which some possess synthase activities, and other possess hydrolytic activities and would be considered as peptipdoglycan hydrolases. Synergistic activity within the PBP family and with other peptidoglycan serine hydrolase enzymes, results in the polymerization and subsequent crosslinking of the glycan strands forming the murein wall (Sauvage et al., 2008). The selective binding therefore of the different β-lactams to the PBP interferes with peptidoglycan biosynthesis. The affinity of the different PBPs differs between the β-lactams resulting in varied effects on the cell morphology as demonstrated in studies involving *E. coli*, a Gram-negative bacillus (Spratt, 1975).

3.7.7 Inhibition of Lysozyme

Bearing in mind the fact that this compound may possess activity against bacterial serine hydrolases, this assay aimed to extend the activity of LY2183240 against lysozyme. Lysozyme belongs to the hydrolase enzymatic class that catalyzes the breakdown of peptidoglycan polymers of bacterial cell wall at the bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine residues, therefore, lysing bacterial cells. The lysing activity of lysozyme is essentially focused on Gram-positive bacteria, as the target (peptidoglycan) is freely accessible to the enzyme, dissimilar to that of Gram-negative microorganisms, which is protected by the lipopolysaccharide layer of the outer membrane (Benkerroum, 2008).

The course of lysis of *M. lysodeikficus* cells (0.1 %) by lysozyme (400 units/mL) at pH 7.2 and the ability of the compound LY2183240 to inhibit the enzymatic activity was assessed (Figure 3.11). The results showed that the lysozyme had the ability to lyse 44 % of *M. lysodeikticus* cells in 30 minutes. When LY2183240 was present in the solution, at the concentrations of 170 mM, only 22 % of cell lysis was achieved in the same period ($p < 0.05$). Imidazole, used as a positive control, exhibited a total inhibition of the cell lysis with time ($p < 0.05$) (Shinitzky et al., 1966).

The moderate inhibitory activity of lysozyme by LY2183240 observed in this study may be related to the fact that this compound possesses a wide range inhibition effect among hydrolases and more assays were carried on to understand this effect.

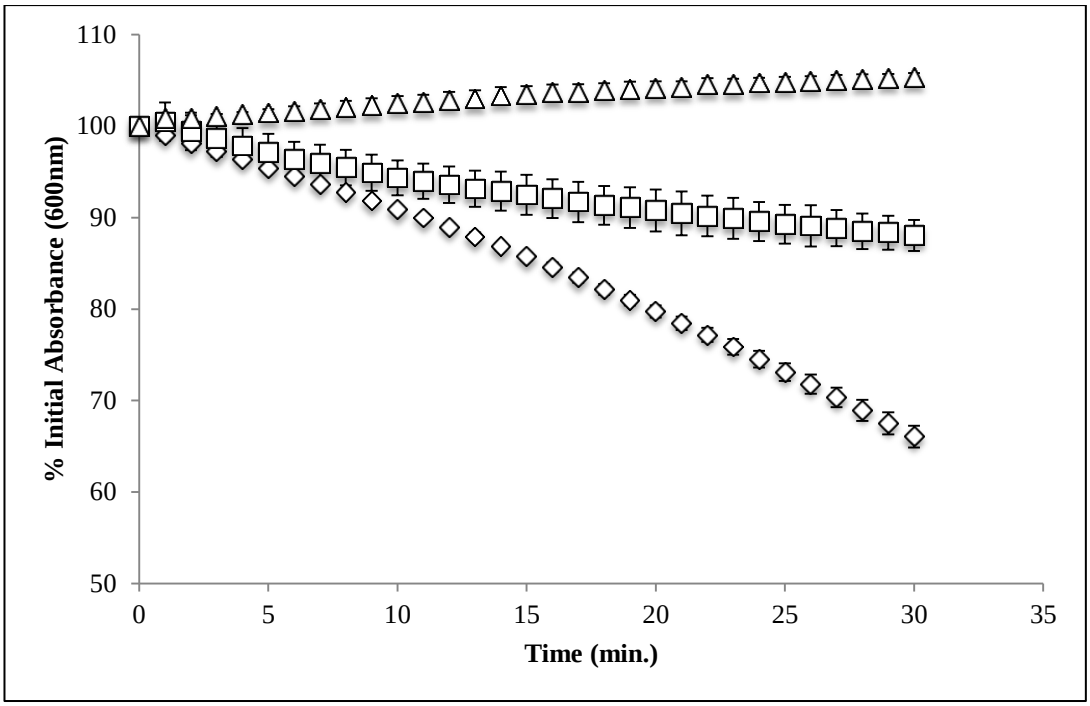


Figure 3.11. The effect of LY2183240 on lysozyme-mediated lysis of *Micrococcus lysodeikticus* cells. The graph illustrates a concentration of 400 units/mL lysozyme (◇, negative control) in combination with LY2183240 (170 mM) (□) and imidazole (170 mM) (△, positive control). Turbidity measurements were taken at 1 minute intervals for 30 minutes. The effect on the *M. lysodeikticus* cells was plotted with Microsoft Excel software. Data represents experiments performed in triplicate.

3.7.8 Modulation of Triton X-100-Induced Cell Lysis

Autolysins are peptidoglycan (murein) hydrolases able to digest the murein of the bacteria that produce them (Korsak et al., 2005). This class of enzymes is omnipresent amongst bacteria (Ghuysen et al., 1966; Mani et al., 1993; Shockman & Höltje, 1994; Foster et al., 2000; Korsak et al., 2005). Additionally, several important roles have been ascribed over the years, including action as a pacemaker for cell wall growth, cell separation, assistance in the murein turnover, lysis initiated by cell wall-active antibiotics, formation of flagella, and bacterial pathogenicity (Goodell, 1985; Mani et al., 1993; Höltje, 1995; García et al., 1999; Korsak et al., 2005).

Some authors claim that the autolytic activity may be stimulated by lysozyme (Wecke et al., 1982), cationic peptides (Wecke et al., 1986), proteolytic enzymes (Ved et al., 1984), and detergents, including Triton X-100 (Raychaudhuri & Chatterjee, 1985), among others. It is believed that the regulation of autolysin

activity happens most frequently at the post translational level, comprising specific activation of the enzyme by substrate modification, topological restriction of enzyme distribution in the cell wall, and control at the site of export (Höltje & Tuomanen, 1991; Mani et al., 1993).

In this study, Triton X-100, a nonionic surfactant capable of inducing enzyme-mediated cell lysis in bacteria, was used in order to verify the effect of LY2183240 on autolysins.

Figure 3.12 shows the lysis of the whole cells from different species when they were re-suspended in buffer containing Triton X-100 (0.05 %). Notwithstanding, under the same conditions and in the presence of LY2183240, a significant reduction of the lysis of all cell types tested was observed ($p < 0.05$).

A marked inhibition by LY2183240 of the whole cell lysis of *S. aureus* (Figure 3.12A) and *B. subtilis* (Figure 3.12B) was noted; the same organisms that were inhibited by the compound. However, LY2183240 did not show antimicrobial activity against *E. faecalis*, but it was capable of slightly limiting the lysis in this case (Figure 3.12C). In the same way, LY2183240 did not demonstrate antimicrobial activity against Gram-negative bacteria, although despite this, inhibition of the lysing activity of *E. coli* cells induced by Triton X-100 was evident (Figure 3.12D).

As potentially suicidal enzymes, autolysins must be strictly controlled by the cell. Some studies suggest that the mechanisms employed to control autolysin activities are ineffective when murein synthesis is affected or inhibited. A classic example is the penicillin effect that results in the induction of cell lysis (Höltje, 1995).

The results obtained in this assay indicate that LY2183240 has a significant impact on the cell lysis of at least three different types of bacteria. The mode of action may be related with inhibition of cell wall hydrolases, but further studies are needed to confirm this.

It should be stressed that the capacity of LY2183240 to inhibit cell lysis of both Gram-positive and Gram-negative bacteria does not correlate with the antimicrobial spectrum of LY2183240 activity.

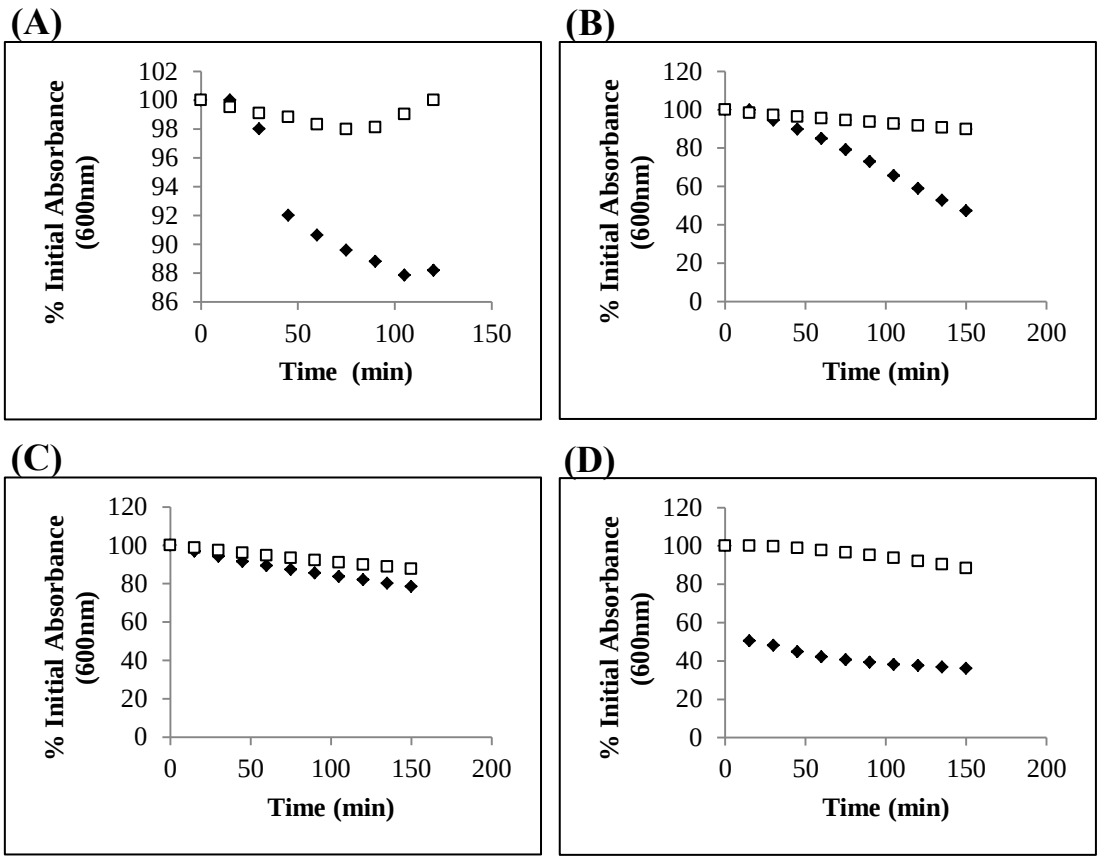


Figure 3.12. Autolysis of whole cells re-suspended in 0.05M Tris-HCl (pH 7.2) containing 0.05% Triton X-100 and incubated at 37°C. Exponential-phase cultures of *Staphylococcus aureus 12981* (A), *Bacillus subtilis* (B), *Enterococcus faecalis 12697* (C), and *Escherichia coli* 10418 (D) with Triton™ X-100 (control; black diamond) and with LY2183240 mixed with Triton™ X-100 (white square). Changes in A_{600} were measured at 10-minute intervals for 120 minutes. Data represents experiments preformed in triplicate.

3.7.9 Detection of Lytic Activity in SDS-PAGE Gels

In order to attempt to identify the specific peptidoglycan hydrolases that were inhibited by LY2183240, SDS-PAGE was employed with *M. lysodeikticus* cells as a substrate challenged with an extracellular extract from *S. aureus*. The total protein profile of the extract is shown in Figure 3.13. The protein profile demonstrated multiple bands with molecular size range from 11 to 58 kDa (Figure 3.13).

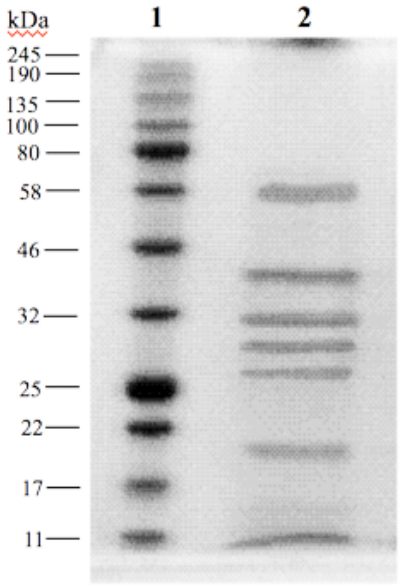


Figure 3.13. Protein analysis by SDS-10% PAGE. Extracellular enzyme extract prepared from *S. aureus* 12981 was separated by SDS-10% PAGE and proteins were visualized after staining with coomassie blue dye. A protein standard (broad range) was loaded in lane 1, and the extracellular extract was loaded in lane 2.

The extract exhibited at least three bacteriolytic enzyme bands (Figure 3.14, lane 2), 20 kDa, 36 kDa, and marked activity associated with a band around 25 kDa. On the other hand, in the lane 3, when LY2183240 was added, a partial inhibition of the lysis of *M. lysodeikticus* cells by the *S. aureus* extracellular proteins (20, 25 and 36 kDa) was noted. Comparably, imidazole demonstrated a similar activity with an apparent reduction of lysis associated with same proteins (Figure 3.14, lane 4).

Alexander and Cravatt (2006) worked on the identification and characterization of the enzymes inactivated by LY2183240, analyzing tissues from mice with an advanced functional proteomic platform referred to as activity-based protein profiling (ABPP)-multidimensional protein identification technology. More than 40 serine hydrolases were identified in the mice brain and they demonstrated that a number of these enzymes were partially or completely inhibited by LY2183240 *in vivo*. In this competitive (ABPP) screen, the selectivity of an inhibitor for members of the serine hydrolase class is determined by measuring reductions in fluorophosphonate-rhodamine labeling intensity for many enzymes in parallel. Remarkably, the fluorophosphonate-labeling of several enzymes was blocked in a concentration-dependent manner by LY2183240, including FAAH, the

uncharacterized hydrolase called KIAA1363, and multiple proteins between the molecular masses of 25-35 kDa.

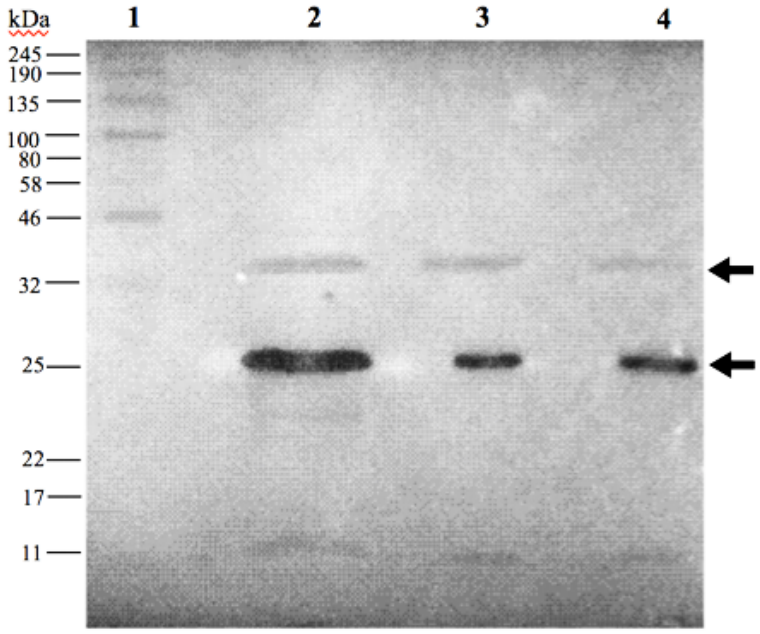


Figure 3.14. Zymogram of the extracellular extract from *S. aureus* 12981 mixed with LY2183240 or imidazole, separated by SDS-PAGE with 0.1% SDS. Equivalent amounts of protein were loaded in each lane. After electrophoresis, proteins were re-natured by overnight treatment with 25 mM Tris - HCl (pH 8.0) containing 1% Triton™ X-100. Lanes: 1, protein standard; 2, extracellular extract (control); 3, extracellular extract + LY2183240 (128 µg/mL); 4, extracellular extract + imidazole (128 µg/mL). The arrows show the lytic enzyme bands of the extracellular extract from *S. aureus* 12981.

Several bacterial autolysins have been characterized using cell walls from *Micrococcus* spp. as a substrate; such assays are frequently employed due to their high level of sensitivity (Ghuysen, 1968; Takahara et al., 1974; Williamson & Ward, 1979). This tactic, utilizing *Micrococcus luteus* cells, has been previously incorporated into polyacrylamide gels to permit detection of various lytic enzymes (Audy et al., 1989).

There are several reports of identified and well-characterized staphylococcal extracellular peptidoglycan hydrolases (Sugai, 1997). Specific to *S. aureus*, Brunskill & Bayles (1996) described an operon consisting of two LytSR-regulated genes. The sequence and mutagenesis studies of these genes suggested that lrgB might encode a protein having murein hydrolase activity. Further, zymographic analysis of this strain revealed the presence of a lytic enzyme band with a size of 25.1 kDa. In another

report, Mani et al. (1993) demonstrated the presence of a 36-kDa bacteriolytic protein in *S. aureus*. Zymographic analyses of a bacteriolytic-compromised mutant revealed the lack of all bacteriolytic bands except the 36-kDa-band when compared with wild type strains. The authors suggested that it probably regulates the expression or processing of autolysins in *S. aureus* and this single lytic enzyme band present in the mutants may be essential for cell morphogenesis.

The bacteriolytic proteins observed in Figure 3.14, may be similar to the murein hydrolases described in these former reports (Mani et al., 1993; Bruskell and Bayles, 1996) and furthermore, compound LY2183240 may inhibit them. However, whether these bacteriolytic proteins identified in this study correspond to lytic enzymes, described in previous studies still awaits definitive determination.

3.7.10 Effect of LY218320 Mixture on AmpC β-Lactamases from *E. coli* G69 and *C. freundii* 382010

β-lactamases are a major cause of bacterial resistance to penicillins and cephalosporins. There are several studies, especially conducted in the 1990's that suggest a close link between β-lactamase induction and the recycling of released muropeptides from the bacterial peptidoglycan (Höltje et al., 1994; Jacobs et al., 1994; Korsak et al., 2005). β-lactam-mediated inhibition of peptidoglycan synthesis stimulates cell wall hydrolases to release muropeptides, particularly anhydro-muropeptides, from the cell wall. AmpG is a transporter responsible for the uptake into the cell of these released muropeptides, and consequently a mutant unable to express AmpG is therefore incapable of recycling the cell wall components and concurrently β-lactamase expression is not inducible by a β-lactam. The cytosolic AmpD amidase degrades the above muropeptides and derivative muropeptides, releasing the tripeptide from cytosolic muropeptides brought into the cell via AmpG (Höltje & Tuomanen, 1991). Mutants inept to produce AmpD are blocked in a cytosolic step for cell wall recycling and accumulate extensive amounts of cytosolic murein-tripeptide. It is considered that cytosolic muropeptides can act as ligands for the β-lactamase regulator AmpR to activate expression of β-lactamase. AmpD mutants, therefore, essentially overproduce the chromosomal β-lactamase and are β-lactam resistant. In wild-type strains, β-lactams that result in an increased cell wall cessation will trigger a rise in the cytosol of muropeptides and give rise to β-

lactamase induction. Mutants affected in the ampD gene can be selected during third-generation cephalosporin-mediated treatment, which are non-inducible, but overexpress β-lactamase in large quantities (Normark, 1995; Stapleton et al., 1995; Stapleton et al., 1999). Once these mutants are deficient in a functional cell wall recycling system they may be at a competitive disadvantage compared to wild-type strains in the absence of selection. Nevertheless, since muropeptides may act as cytotoxins, especially for respiratory epithelial cells, AmpD mutants due to their large accumulation of murein-tripeptide may be altered in their pathogenic properties when compared to wild-type cells possessing a normal cell wall recycling system (Normark, 1995).

Many Gram-negative bacteria, including *C. freundii* 382010, express a chromosomally encoded AmpC β-lactamase, which is inducible by many β-lactams, for instance meropenem (Sykes & Matthew, 1976). Since β-lactam induction is dependent upon cell wall hydrolase activity, if LY2183240 blocks β-lactamase induction in the presence of an inducer such as meropenem, this could be further evidence that LY2183240 inhibits cell wall hydrolase activity.

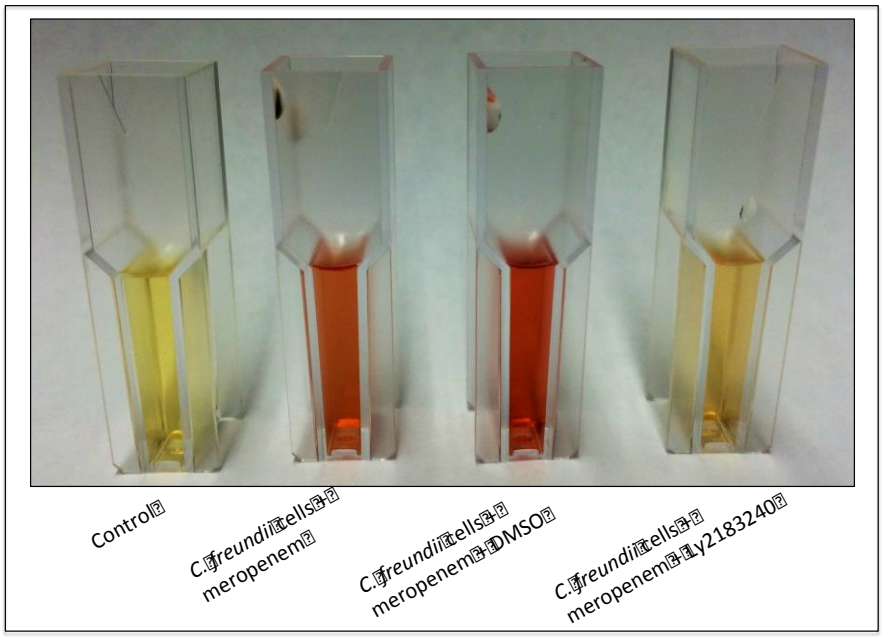


Figure 3.15. β -Lactamase induction assay using *Citrobacter freundii* in the presence of meropenem, DMSO and LY2183240. β -Lactamase activity is indicated by a red color after 1 - 3 minute reaction after the addition of nitrocefin. Photo: P. E. de Resende.

β-Lactamase activity can be detected utilizing nitrocefin. Nitrocefin is a chromogenic cephalosporin that changes from yellow to red on hydrolysis, providing a sensitive test for most β-lactamases with a few exceptions (Livermore, 1995).

It was observed that the antibacterial agent meropenem was capable of inducing significant β-lactamase production in *Citrobacter freundii* cells in comparison to the control in the absence of this drug (Figure 3.16). Conversely, when LY2183240 was incubated with *C. freundii* and meropenem for a 1-hour period, a reduction of the β-lactamase production was noted ($p < 0.05$) (Figure 3.16).

Since β-lactamase is released from cells as they die during stationary phase, supernatant (growth media with cells removed) from an overnight culture can be used as a source of the enzyme. An assay was performed using the supernatant of the *C. freundii* culture in order to understand the mechanism of action of the potential inhibitory activity of LY2183240. Figure 3.16 illustrates that β-lactamase can be detected within *C. freundii* supernatant after induction with meropenem. As in the previous assay, meropenem showed evident induction of β-lactamase production.

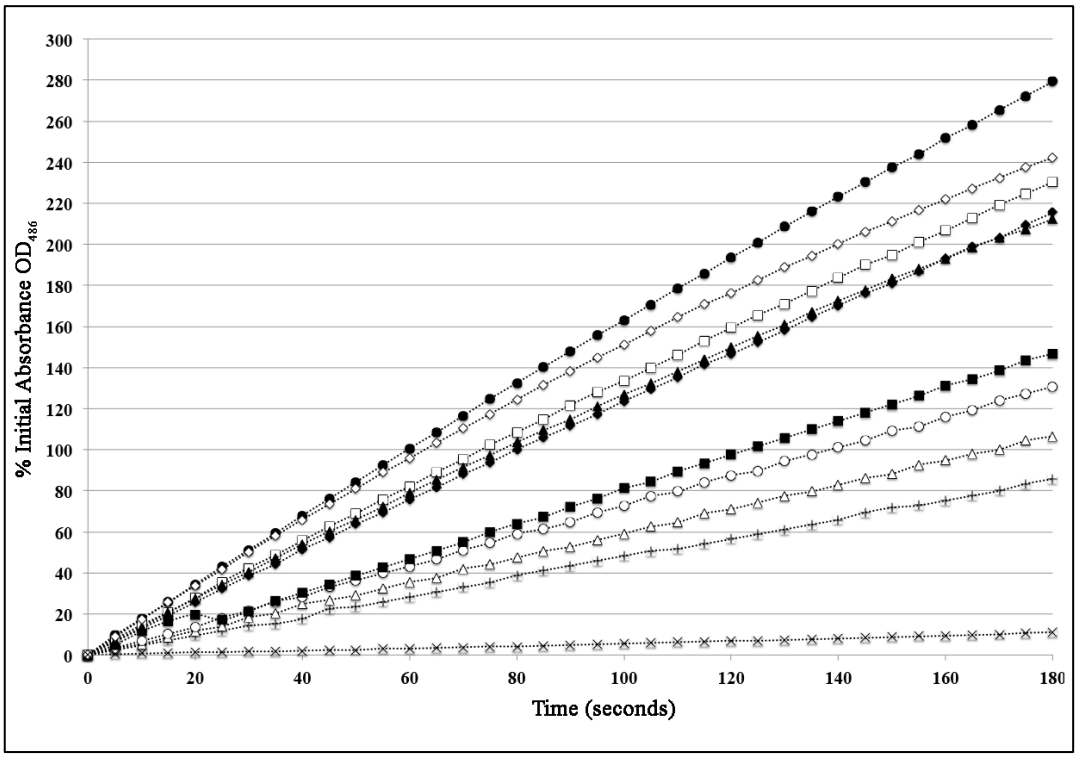


Figure 3.16. β-lactamase in *C. freundii* 382010. β-lactamase activity was determined using a nitrocefin-based assay on supernatant of *C. freundii* cultures that had been incubated with meropenem alone (●), LY2183240 mixture 420 μM (+), 200 μM (△), 100 μM (○), 50 μM (■), 25 μM (◆), 15 μM (□), 6.5 μM (▲), and 3 μM (◇), and (×) shows β-lactamase activity in a control not incubated with meropenem.

Furthermore, LY2183240 apparently inhibited β-lactamase production ($p < 0.05$). Notwithstanding, in this particular case, since no cells were present in the solution, LY2183240 could potentially be acting as a β-lactamase inhibitor rather than preventing β-lactamase induction per se.

As described in the literature, β-lactamase inhibitors present different strategies to combat resistance mediated by these enzymes. One of the most efficacious approaches is the use of agents able to bind at the active site, which have previously been β-lactam-based (Drawz & Bonomo, 2010). This can be achieved by reversible or irreversible substrates that bind the enzyme with high affinity with unfavourable steric interactions as an acyl-enzyme or as mechanism-based "suicide inhibitors" that can permanently inactivate the β-lactamase through secondary chemical reactions within the enzyme active site. Some examples of mechanism-based inhibitors are clavulanic acid, sulbactam, and tazobactam, which are recognized as a substrate by the β-lactamase, bonding them covalently to produce chemical modifications that cause irreversible attachment to the enzyme (Pérez-Llarena & Bou, 2009). The fact that LY2183240 reduced the β-lactamase activity in the supernatant of *C. freundii* may suggest a direct interaction with the enzyme like mechanism-based inhibitors.

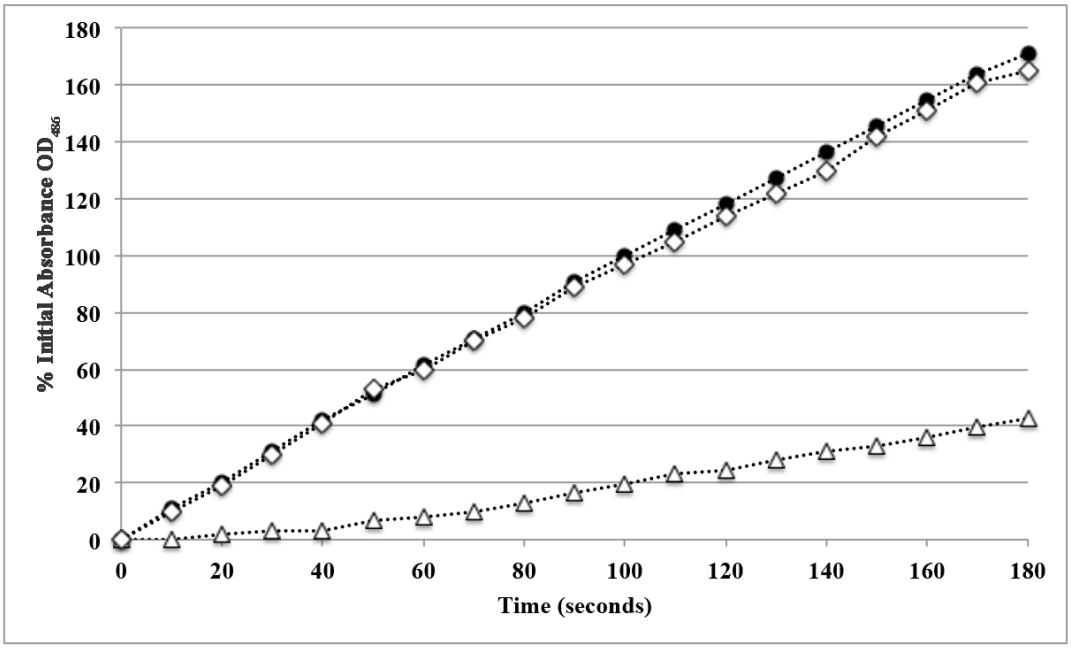


Figure 3.17. The effect of AmpC β-lactamase from *E. coli* G69 (●, control) and in presence of 420 μM LY2183240 mixture (△). DSMO was used as a control (◇).

Figure 3.17 illustrates the influence of LY2183240 towards AmpC from *E. coli* G69,

a multi-drug resistant isolate that expresses the plasmid encoded class C β-lactamase, which is non-inducible. The supernatant of the *E. coli* G69 was collected and processed similarly to *C. freundii*, although without induction. Similar to *C. freundii*, β-lactamase activity was detectable with a relevant rise in the percentage of initial absorbance of nitrocefin at 486 nm, but in presence of LY2183240 at 420 μM, a significant reduction in the enzyme activity was observed (Figure 3.17).

Again, the solvent used to dissolve the samples, DMSO, showed no effect on enzyme activity.

It is generally agreed that cell wall metabolism and β-lactamase induction have a complex biochemistry and the signaling pathways for the induction of these enzymes offer a broad array of promising targets for the discovery of novel antibacterial drugs as well as the understanding of the biochemical processes. According with the previous results presented in this study, LY2183240 exhibited a potential influence in the activity of bacterial hydrolases. Although the specific mechanism of action is still unknown, the activities found here are intriguing and deserve more exploration. To further understand the mode of action of LY2183240 towards β-lactamases and others bacterial hydrolases additional biochemical and molecular studies are necessary and will be discussed in the next chapter.

Although this part of the study has successfully demonstrated that LY2183240 reduced AmpC β-lactamases activity from Gram-negative bacteria, it has certain limitations in terms of enzyme standardization and sample purity. At this stage of the project LY2183240 was being used as a mixture due to difficulties in isolating both regioisomers. Nevertheless, at this point these results can be considered preliminary and encouraging, leading this study to pursue this intriguing activity of the compound towards bacterial serine hydrolases.

In order to verify to what extent this compound affects other β-lactamases, the next section moves on to examine the potential activity towards class A penicillinases.

3.7.11 Inhibition of Class A β-Lactamase (TEM-1)

Class A β-lactamases are, in general, susceptible to commercially available β-lactamase inhibitors such as clavulanic acid, and tazobactam. Nonetheless, there is an increasing number of this class of β-lactamases in *E. coli* and *K. pneumoniae*, as well as the occurrence of these enzymes in other significant pathogens, that are refractory

to inhibitors, leading to the development of extended-spectrum cephalosporins, carbapenems, and monobactams (Drawz et al., 2010).

Subsequent to the finding of the inhibitory activity of LY2183240 against AmpC β-lactamases from *E. coli* and *C. freundii*, the potential activity of this compound towards class A β-lactamase was assessed. The TEM-1 β-lactamase was selected for two main reasons. The first is because TEM-1 is a standard reference for class A and commercially available and easily accessible. The second is because the enzyme is one of the most frequently encountered plasmid-mediated β-lactamases identified in clinical isolates of *E. coli* (Cooksey et al., 1990). LY21832440 mixture was evaluated against TEM-1, and clavulanic acid was used as a reference inhibitor (Figure 3.18).

It was observed that in presence of the chromogenic substrate nitrocefin, TEM-1 showed a gradual activity increase in a time-dependent fashion. In addition, DMSO (sample dissolution solvent) had no significant effect on enzyme activity.

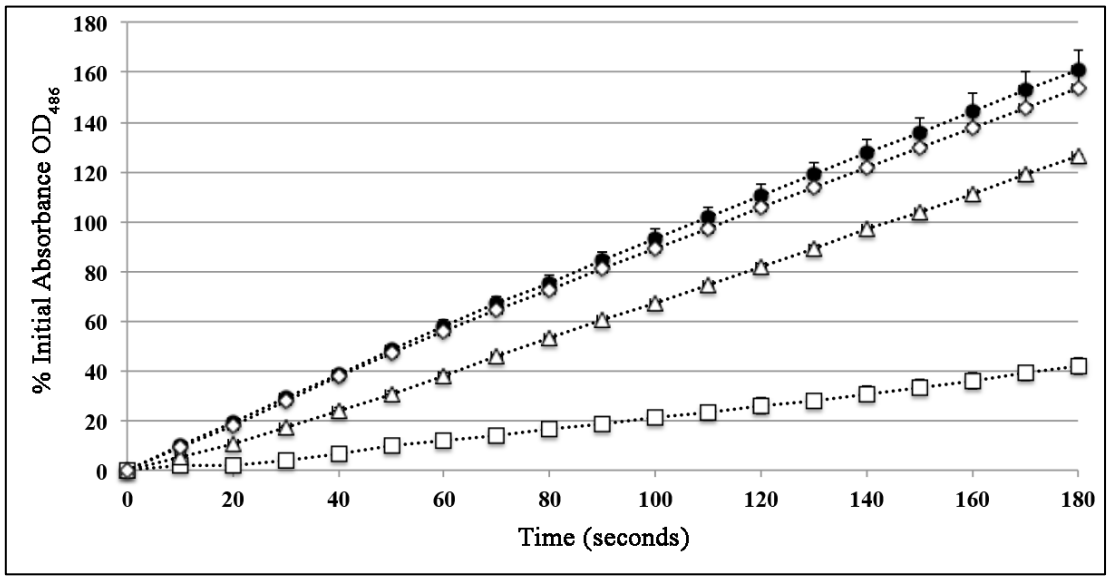


Figure 3.18. The activity of TEM-1 β-lactamase (2.5 μM) using nitrocefin (100 μM) as a substrate (●, control) and in the presence of 420 μM LY2183240 mixture (△) or 10 μM of clavulanic acid (□). DSMO was used as a solvent control (◇).

Unpredictably, the LY2183240 exhibited considerably less potency towards TEM-1 when compared to AmpC β-lactamases. At the highest concentration of 420 μM, LY2183240 demonstrated a minor reduction in the class A enzyme activity (Figure 3.18). Conversely, at the concentration of 10 μM, clavulanic acid dramatically

reduced the β-lactamase activity. This result is in line with former findings, where clavulanic acid revealed a potent activity against TEM-1. Many studies demonstrated that the inhibition constant (K_i) of clavulanic acid is 0.1 μM towards TEM-1 (Chaibi et al., 1998; Imtiaz et al., 1994; Naumovski et al., 1992). Moreover, according to Bush (1988) it is necessary for only 160 clavulanate molecules to completely inactivate TEM-1, indicating a significant potency against this β-lactamase.

The fact that LY2183240 mixture presented a stronger effect against class C than class A suggests that this compound has a considerable selectivity towards β-lactamases. As revealed by Alexander and Cravatt (2006), LY2183240 possess a promiscuous activity against several serine hydrolases detected through a functional proteomic screen. Nevertheless, Ortar and coworkers (2008) agreed only in part with the conclusions drawn by these authors. They found that out of the 17 tetrazoles synthesized and screened, four compounds presented potent activity for FAAH selectively over all other targets tested, corroborating with the selectiveness presented in this study.

Admittedly, a major source of unreliability in this part of the study is the fact that LY2183240 is a mixture potentially containing the regioisomers 1,5-LY (~50 %) and 2,5-LY (~39 %) as aforementioned in the chemical characterisation. Hence, the proportion and content of the mixture may influence directly the inhibitory activity against β-lactamases.

Therefore, Chapter 4 of this thesis is mainly dedicated to addressing the biological and chemical evaluation of LY2183240 regioisomers 1,5 and 2,5 independently as well as pursuing the potential modes of action of the activities indicated in this chapter.

3.7.12 Antimicrobial Potentiation Activity of LY2183240

The major aim of this assay is to evaluate the potential resistance-modifying and synergy effects of LY2183240 with amoxicillin and cefoxitin against antimicrobial resistant Gram-negative organisms.

Although the end-points were not determined, LY2183240 showed no significant antibiotic potentiation effect against the strains tested (Table 9). The MIC values for the standard antimicrobial agents used were the same with or without LY2183240. There are some possible explanations for these results. Firstly, the presence of efflux

pumps system strongly limit the intracellular concentration of antimicrobial agents (Poole, 2005; L. J. V. Piddock, 2006; Poole, 2007; Li & Nikaido, 2009). The genes and proteins of this system can be present in both antibiotic-susceptible and antibiotic-resistant bacteria. There are at least two mechanisms for a resistant strain: (1) expression of the efflux pump protein is augmented; and (2) the protein contains an amino acid substitution(s) that makes the protein more effective at drug export. Whichever the case, the intracellular concentration of the substrate antimicrobial is reduced and the organism becomes less susceptible to that particular agent (L. J. V. Piddock, 2006).

The multi-drug resistant clinical isolate *E. coli* used in this study has not been completely characterised although the organism is likely to have several efflux pumps. In addition, this strain carries plasmids that encodes class A and C β-lactamases, also contributing towards the antimicrobial resistance phenotype (Stapleton et al 1999). Likewise, the *C. freundii* strain employed intrinsically produced class C β-lactamases. The potential activity of LY2183240 against β-lactamases will be investigated more extensively in Chapter 4.

Table 9 Antimicrobial potentiation activity of LY2183240 towards Gram-negative bacteria.

Bacteria	***E. coli* G69**		***C. freundii* 382010**	
Antibiotic/compound	AMX	AMX+2,5-LY	CEF	CEF+2,5-LY
MIC (μg/mL)	> 512	> 512	> 256	> 256

AMX, amoxicillin; CEF, cefoxitin.

Another possible reason is that Gram-negatives are intrinsically resistant to certain compounds due to the presence of the outer membrane. This membrane plays a fundamental role of providing an extra layer of protection to the Gram-negative organisms (Delcour, 2009). The outer membrane consists of an inner leaflet comprising phospholipids and an outer leaflet containing the lipid A moiety of lipopolysaccharides (Kumar & Schweizer, 2005). This composition gives impermeability to many substrates and transportation across the outer membrane is achieved by porin proteins that form water-filled channels.

The possibility of LY2183240 being prevented of entering in Gram-negative bacteria will be investigated further and discussed in Chapter 4.

3.8 Conclusions

The data in this chapter demonstrate that none of the plants extracts evaluated had useful direct antimicrobial activity. The discrepancies in the MIC values when compared with previous studies may be due to differences in the methods used to extract and determine the MICs, or to differences in the characteristics of the plants, such as their origins. The lack of antimicrobial activities with any of the extracts suggests that they may not be useful as leads for new antibiotics. Nonetheless, more studies are necessary to confirm whether they have any other useful properties such as resistance modulatory activities.

With regards to LY2183240, collectively the results show potent growth inhibitory activity against certain Gram-positive bacteria, including *S. aureus* 12981 (MSSA), *S. aureus* 13373 (MRSA) and *B. subtilis*. Additionally, chemical characterisation reveals that the sample evaluated consisted of a mixture of two major compounds, conceivably being regioisomers of LY2183240, as formerly reported in the literature (Ortar et al., 2007; Ortar et al., 2008; Asada et al., 2015).

One of the purposes of this study was to extend early observations of the inhibition of serine hydrolases in eukaryotes and to provide a basis for the potential antimicrobial effectiveness of the compound.

The inhibition of cell lysis stimulated by either the activity of lysozyme, Triton^{TM} X-100 or penicillin G, by LY2183240 suggests the compound inhibits autolysins associated with the bacterial peptidoglycan. Autolysins are peptidoglycan hydrolases that lyse the cell in such a way that the integrity of the murein wall is damaged. This class of enzymes is involved in several bacterial processes such as cell division and separation, inhibition of their action would therefore affect the pathogenicity and virulence of the microbe (Smith, Blackman et al. 2000). The presence of multiple autolysins as well as their functional redundancy complicates attempts to identify which enzyme is specifically targeted by LY2183240, if that is indeed the case. In this context, partial inhibition of bacterial hydrolases from *S. aureus* by LY2183240 was shown, with effects on at least three proteins with molecular masses between 25 - 35 kDa. Moreover, LY2183240 was able to significantly inhibit β-lactamases induced by meropenem in *C. freundii* cells, exhibiting a possible link between murein hydrolases and β-lactamases, formerly reported in literature (Höltje et al.,

1994; Jacobs et al., 1994; Korsak et al., 2005). However, the activity against cell wall hydrolases does not correlate with the antimicrobial spectrum of activities per se.

One of the limitations of this first part of the study was the difficulty to obtain a pure compound from a reliable source. Nevertheless, these findings successfully demonstrated some antimicrobial and resistance-modifying effects of LY2183240. Therefore, in Chapter 4 the potential specific targets of the regioisomers of LY2183240 will be explored to gain a better understanding of the antimicrobial mode of action of the compound.

4 CHAPTER 4

Biological and Chemical Evaluation of LY2183240 regioisomers

4.1 Introduction

The pathogens *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species are the leading cause of nosocomial infections throughout the world (Santajit & Indrawattana, 2016). Most of them are multidrug resistant isolates, which is one of the greatest challenges in clinical practice. The emergence of methicillin-resistant *S. aureus* (MRSA), for instance, is a source of concern with millions diagnosed with MRSA infections each year. Another significant mechanism of antibiotic resistance presented by some of these pathogens is the production of enzymes that irreversibly modify and inactivate the antibiotics, such as β-lactamases (Drawz et al., 2010).

As a consequence, research has focused on combatting these mechanisms that bacteria have developed to resist antibacterial agents.

Chapter 3 revealed significant and promising antimicrobial-related activities of LY2183240. Firstly, this simple molecule showed potent direct antimicrobial activity against certain Gram-positive bacteria, especially *S. aureus* including MRSA. Secondly, the compound inhibited class C β-lactamases.

This chapter will explore in detail these different aspects; in this case using pure preparations of LY2183240 regioisomers independently in order to assess the impact changes in the structure of these molecules have towards prokaryotic systems. Former reports in the literature showed that the simple position change of the carbamoyl group from -1,5 to -2,5 in the tetrazole influenced considerably the potency of the activities towards mammalian targets (Ortar et al., 2007; Ortar et al., 2008).

4.2 Objectives

The main objective of this chapter is to evaluate the direct antimicrobial and resistance-modifying properties of each of the two LY2183240 regioisomers.

The specific aims of the study are as follows:

- To determine the minimum inhibitory concentrations (MICs) of both LY2183240 regioisomers.
- To determine the potential target or targets of the antimicrobial activity of LY2183240 regioisomers using different methods and approaches.
- To assess the β-lactamase inhibitory properties via an extensive enzyme kinetic study.
- To determine the IC_{50} and K_i of both LY2183240 regioisomers towards β-lactamases.

4.3 Results and Discussion

4.3.1 Characterisation of LY2183240 Regioisomers

In order to establish the purity of the compounds, as stated by the suppliers, characterisation of both LY2183240 regioisomers was performed by HPLC, NMR and mass spectrometry.

4.3.1.1 HPLC Analysis

The HPLC analysis was implemented as stated on Chapter 2, section 2.3.2.1.2. In addition the LY2183240 mixture was used for comparison purposes.

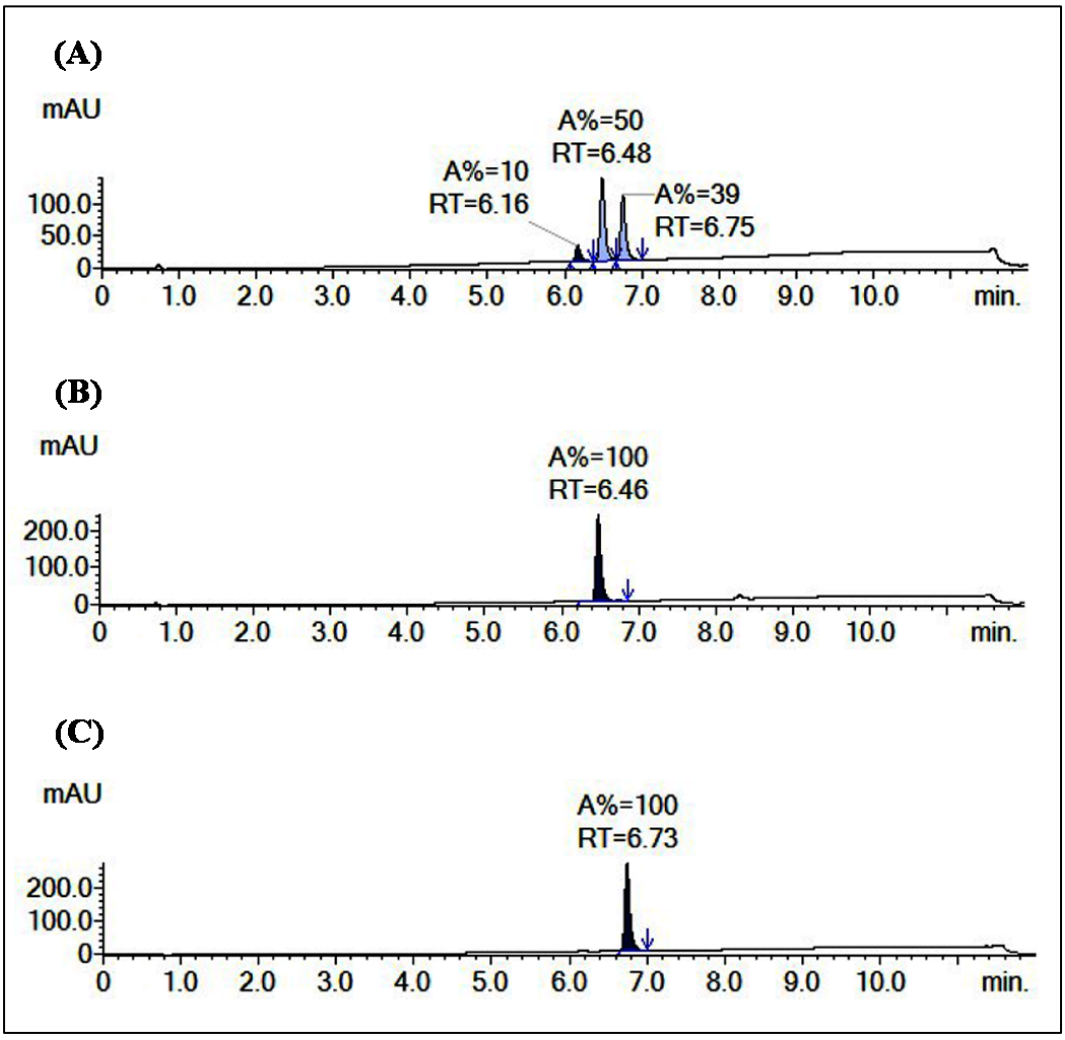


Figure 4.1: HPLC chromatograms of LY2183240. **(A)** LY2183240 mixture; **(B)** 1,5-regioisomer; **(C)** 2,5-regioisomer.

It was possible to confirm the presence of the 1,5-regioisomer in the sample. The chromatogram revealed a single peak with the retention time of 6.46 minutes and an area under the curve of 100 %, suggesting a highly pure sample as stated by the provider Santa Cruz Biotechnology (Figure 4.1B). Comparably, the 2,5-regioisomer exhibited only one peak, with retention time of 6.73 minutes and 100% of area under the curve, indicating a pure sample as well (Figure 4.1C). In addition, when compared with the LY2183240 preparation used in the first part of this project, it was observed that both regioisomers were present in this mixture, having similar retention times to the pure samples (Figure 4.1A). The analysis revealed a content of 50 % and 39 % for 1,5-isomer and 2,5-isomer, respectively. Moreover, an extra minor peak was noted (10 %) with a retention time of 6.16 minutes that could be another potential isomer of LY2183240 or an impurity of the synthesis process.

This complete analysis employing all LY2183240 samples used in this study reiterates the hypothesis of the 2,5-isomer being responsible for the anti-staphylococcal activity found in Chapter 3 and confirmed in the section 4.3.2.1 below.

4.3.1.2 NMR Analysis

Full NMR analysis including ^{1}H, ^{13}C, DEPT, COSY, HMQC, HMBC and NOESY experiments with both LY2183240 regioisomers in methanol-d_4 were performed.

The results of the ^{1}H NMR spectrum for 1,5-LY2183240 were as follows: *d* 2.70 (6 H, *s*), 3.07 (2H, *s*), 4.46 (1H, *t*), 7.34 (2H, *d*), 7.42 (2H, *t*), 7.60 (4H, *d*).

The results of the ^{1}H NMR spectrum for 2,5-LY2183240 were as follows: ^{1}H NMR (400 MHz, methanol-d_4): *d* 3.05 (6 H, *s*), 3.23 (2H, *s*), 4.37 (1H, *t*), 7.32 (2H, *d*), 7.40 (2H, *t*), 7.59 (4H, *d*).

The ^{1}H and ^{13}C NMR data of these compounds were in agreement with published literature (Ortar et al., 2008; Asada et al., 2015). Notwithstanding, a full assignment of the ^{1}H and ^{13}C NMR resonances of both regioisomers was performed. HMQC, HMBC, COSY and NOESY data confirmed the structures. All the spectra of both LY2183240 regioisomers can be found within Appendix section 8.1.

The ^{1}H-NMR resonances of the *N*,*N*-dimethyl $(CH_3)_2$ group were clearly visible within the space correlations between the protons from the two methyl groups in the NOESY analysis (Appendix 8.1). HMBC correlations were observed for both regioisomers from the methylene protons (H2-1") to C-3" and 4" and within the

biphenyl rings, for example from H-3" to C-1", as well as from H-2" to C-4". COSY correlations from H-5" to H-6" and from H-2" to H-3", which in turn coupled to H-4" together with the NOESY coupling between H-1" and H-3", were also noticed and assisted the confirmation of the proton resonances. The essential resonances to differentiate and authenticate these compounds were the ^{13}C resonances of the tetrazole carbons and the carbonyl carbons. The ^{2}J and ^{3}J correlations from the dimethyl protons to the carbonyl carbons and from methylene protons (H-1") to tetrazole carbons (C-5) observed in HMBC spectra of both regioisomers permitted the complete assignment of the ^{13}C resonances. The carbonyl carbons of 1,5-LY2183240 and 2,5-LY2183240, which were both attached to nitrogen atoms of the tetrazole ring revealed ^{13}C resonances at a comparable chemical shift, δ_C 149.3 and δ_C 149.6, respectively (see Figures 8.1A and 8.4A). Furthermore, the CN_4 ^{13}C resonance of the 1,5-regioisomer was noticed at a higher field (δ_C 157.3) when compared to the 2,5-regioisomer (δ_C 166.7) suggesting that the tetrazole carbon of 1,5- was more shielded. In summary, the extensive NMR analysis confirms both regioisomer structures as declared by the chemical suppliers.

4.3.1.3 Mass Spectroscopy Analysis

The mass spectra of both LY2183240 regioisomers showed a peak at *m/z* 306.4 and *m/z* 306.7 for 1,5- and 2,5-, respectively, which corresponded to the chemical formula of $C_{17}H_{17}N_5O$. The other peaks present in the analyses represent the fragmentation pattern formed by the ionization process (Figures 4.3 and 4.5).

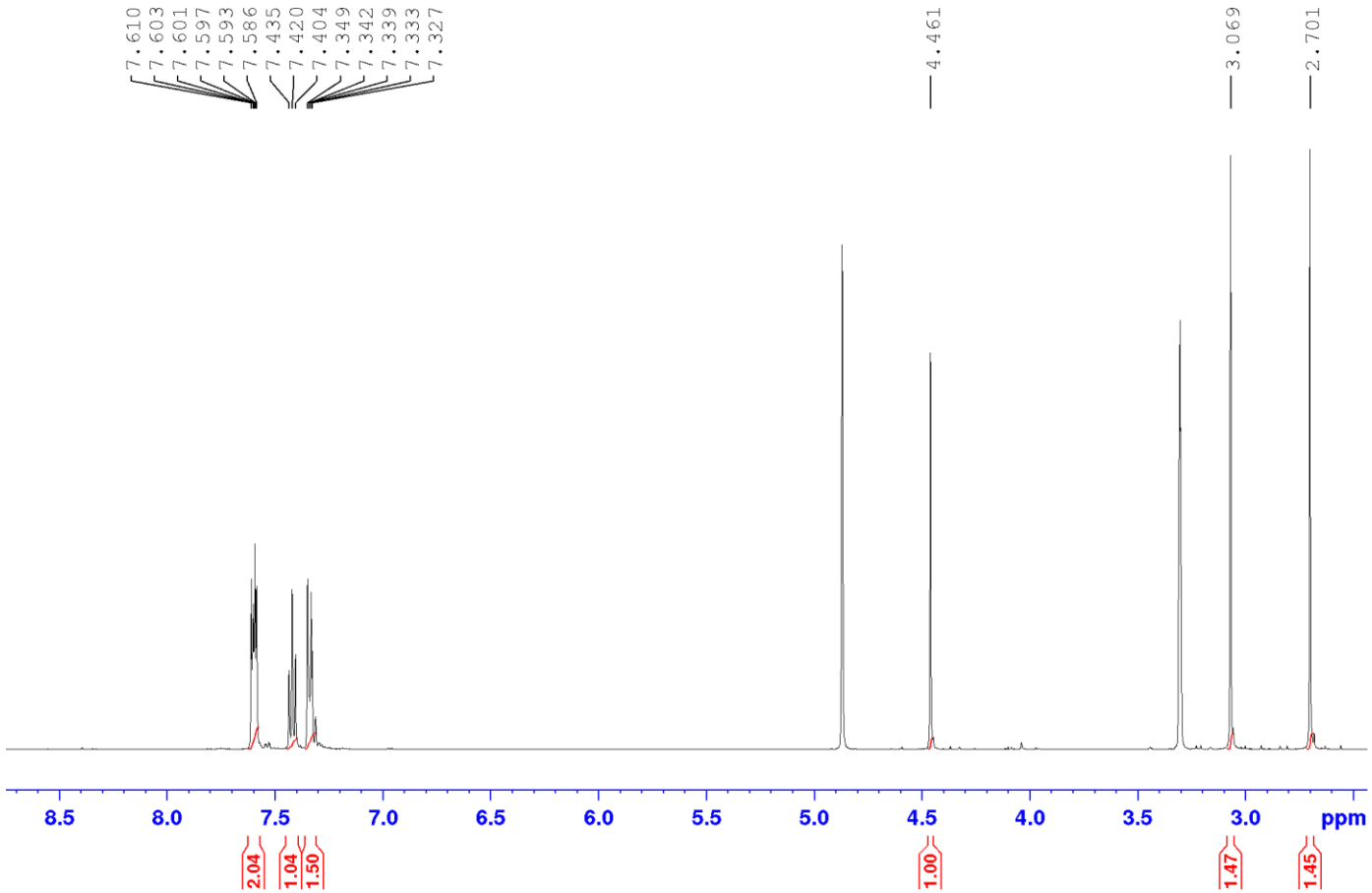


Figure 4.2. ^{1}H NMR spectrum for 1,5-LY2183240, recorded in methanol-d_4, 400MHz.

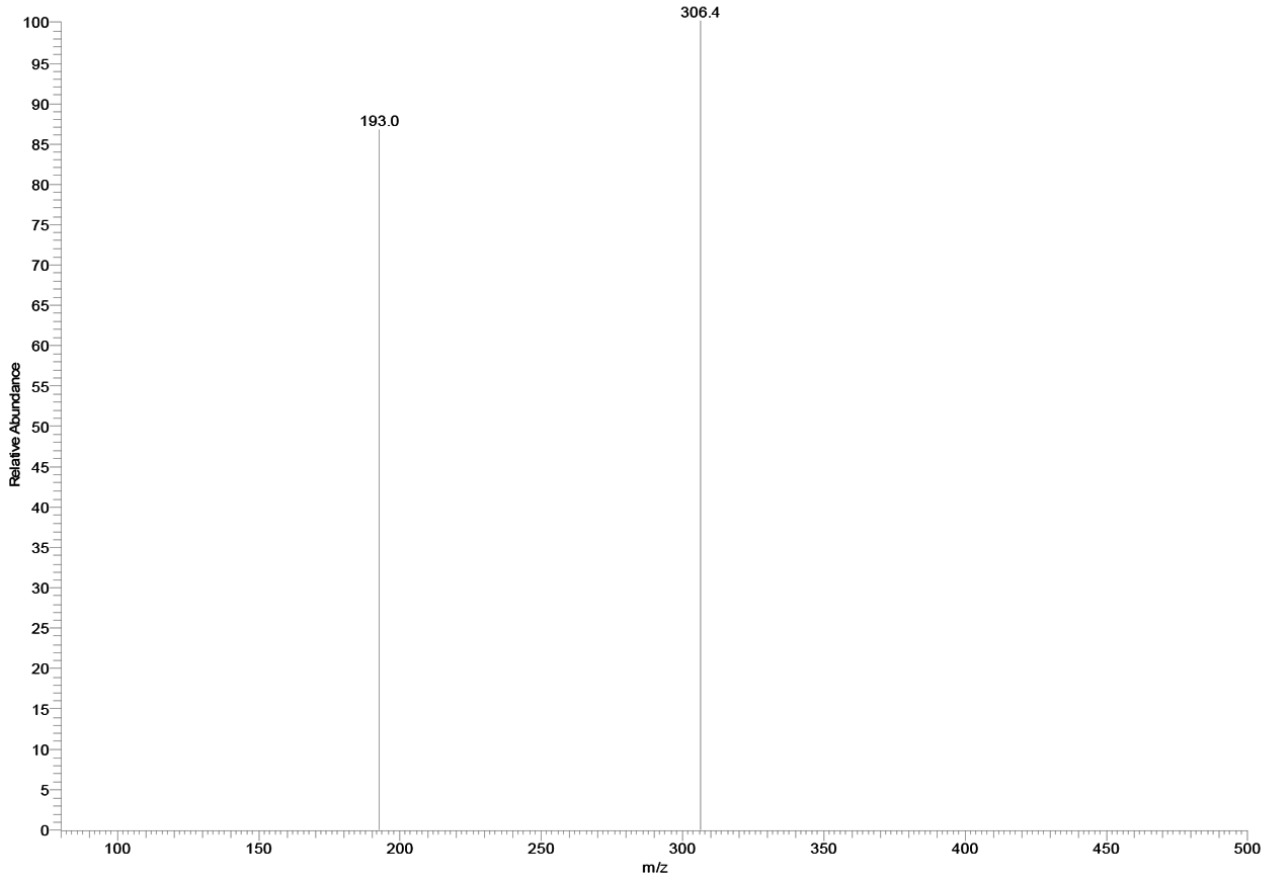


Figure 4.3. ESI-MS/MS spectrum for the isomer 1,5-LY2183240.

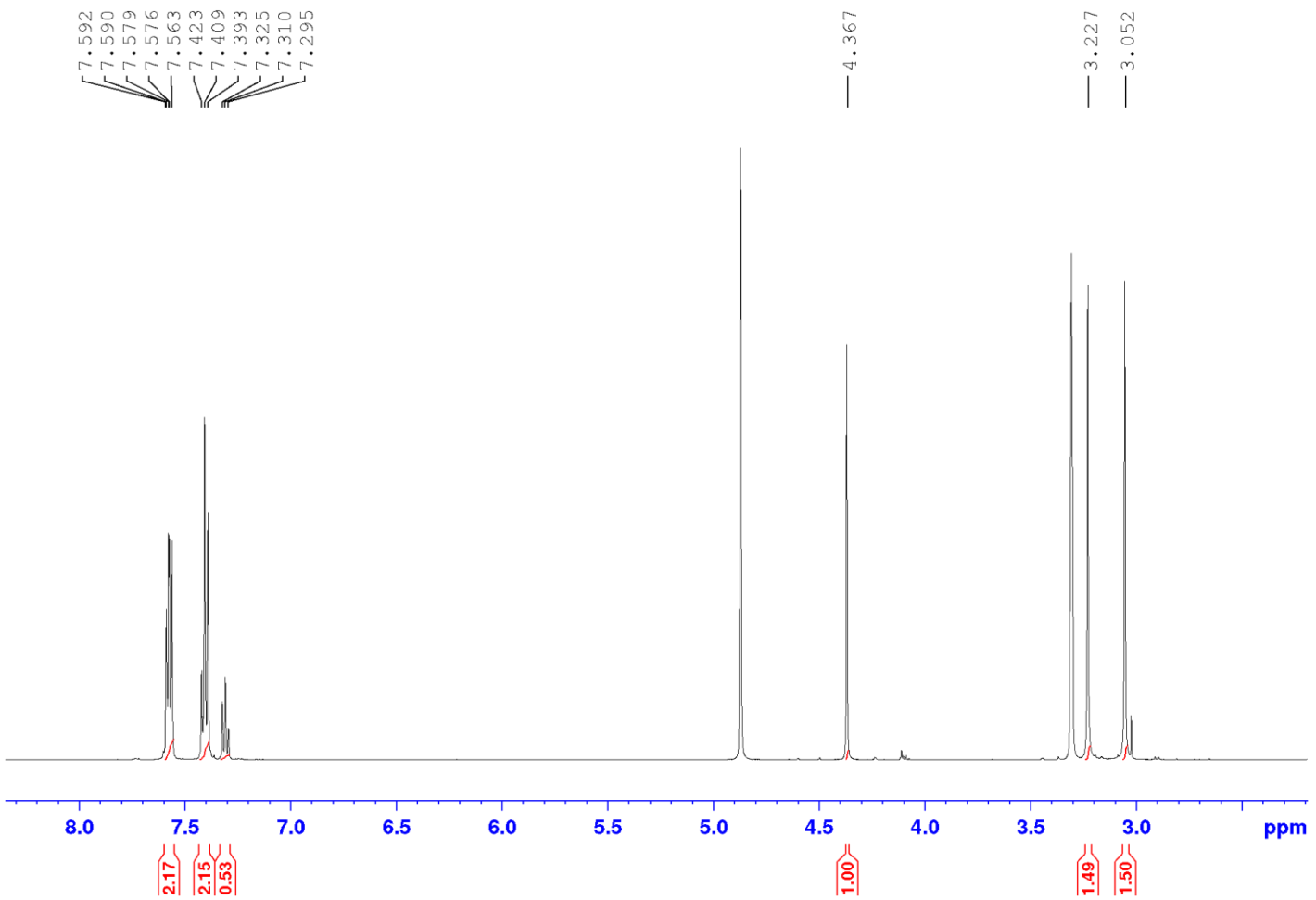


Figure 4.4. ^{1}H NMR spectrum for 2,5-LY2183240, recorded in methanol-d_4, 400MHz.

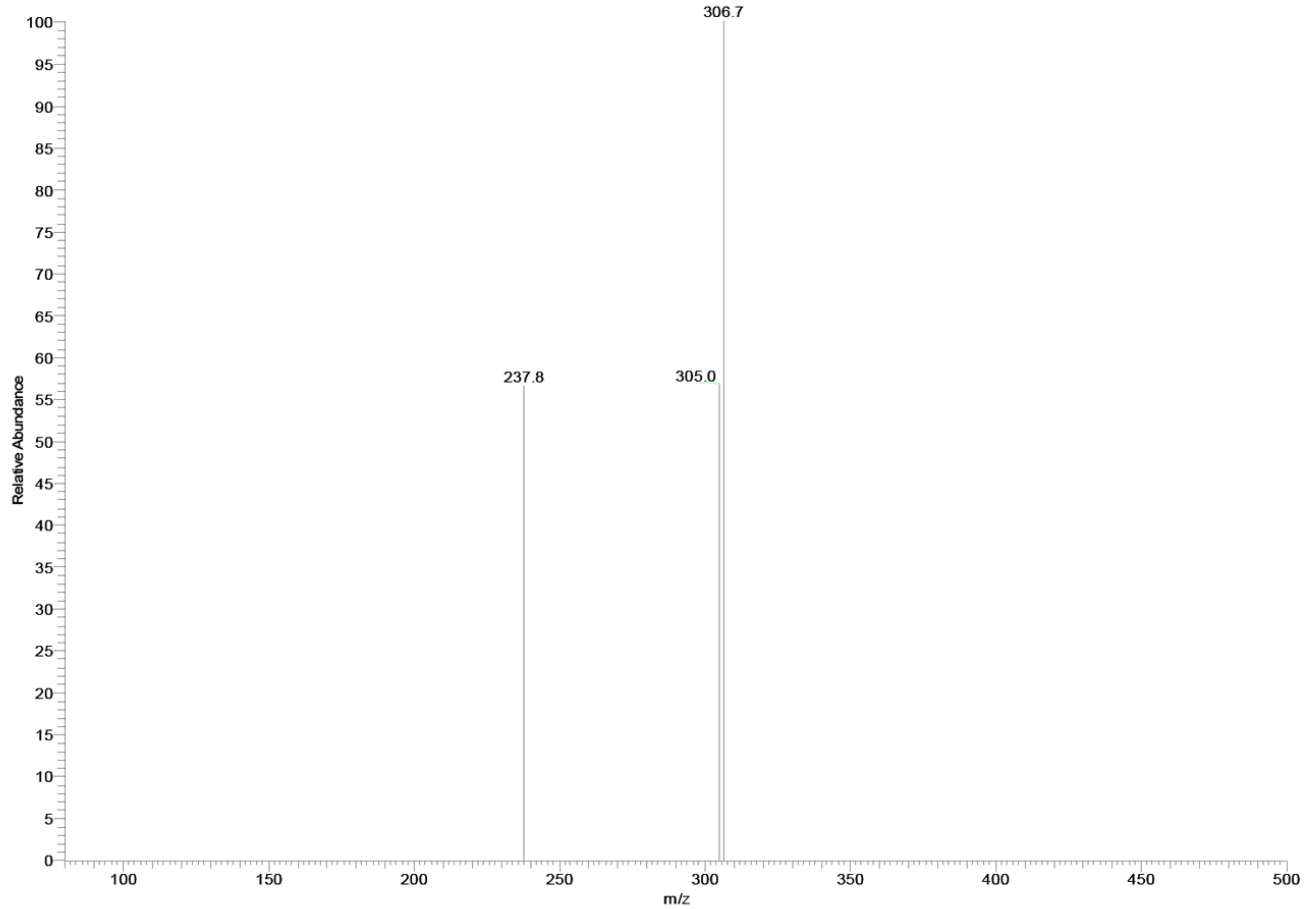


Figure 4.5. ESI-MS/MS spectrum for 2,5-LY2183240.

4.3.2 Biological Evaluation

4.3.2.1 Minimum Inhibitory Concentration

The antimicrobial activities of both regioisomers of LY2183240 were evaluated through the determination of minimum inhibitory concentrations by the broth microdilution method; the results are summarized in Table 10.

Remarkably, only the 2,5-isomer exhibited a potent antimicrobial effect against certain Gram-positive bacteria. Moreover, this compound revealed potent activity towards staphylococcal isolates, including MRSA, with drug MIC values as low as 0.5 μg/mL against a methicillin-resistant clinical isolate. 2,5-LY2183240 had no effect against the Gram-negatives or other Gram-positives tested such as *E. faecalis* strains. The 1,5-regioisomer showed no significant activity against all microorganisms tested, with MIC values higher than 128 μg/mL.

These results strongly suggest that the position of the carbamoyl in the tetrazole plays an essential role in the direct antimicrobial activity observed. As seen in Chapter 3, the LY2183240 mixture, containing both regioisomers, gave rise to bacteriostatic effects against Gram-positives, including *Staphylococcus aureus*. Nevertheless, when the isomers were assessed separately, only the 2,5-isomer showed activity, implying that the 2,5-isomer is responsible for the antimicrobial activity found in the mixture.

As stated before, both LY2183240 isomers present a tetrazole group in the structure (See Figure 1.17). Tetrazole is a class of synthetic organic heterocyclic compounds consisting of a five-member ring of four nitrogen atoms and one carbon atom. In general, substantial evidence has gathered during the past decades demonstrating the countless pharmacological effects of tetrazoles and their derivatives (Mohite PB & Bhaskar VH, 2011).

Table 10. Minimum inhibitory concentrations of LY2183240 regioisomers determined by broth microdilution assay.

Organism	Standard Antibiotic	1,5-LY2183240	2,5-LY2183240
S. aureus 12981	0.125[a]	>128	1 − 2
S. aureus 13373	4[a]	>128	4 − 8
B. subtilis 15	0.125[a]	>128	8 − 16
MRSA 80415G*	>128[a]	128	1 − 2
MRSA 80415R*	>128[a]	128	4 − 8
MRSA 80415S*	>128[a]	>128	0.5 − 1
MRSA 80415K*	>128[a]	>128	2− 4
EMRSA 15	Not determined	>128	2 − 4
RN4220	1[b]	>128	2 − 4
XU212	16[b]	>128	0.5 − 1
S. aureus 1199b	32[b]	>128	8 − 16
S. epidermidis	0.125[a]	>128	2 − 4
E. faecalis 12967	0.5[a]	>128	>128
E. faecalis 13379	4[a]	>128	>128
Stenotrophomonas sp.	1[a]	>128	>128
E. coli NCTC 10418	0.125[b]	>128	>128
E. coli G69*	>128[c]	>128	>128
K. pneumoniae 17	32[c]	>128	>128
Streptococcus pneumoniae	0.125[a]	>128	>128
P. aeruginosa 10662	1[b]	>128	>128

*Clinical isolates; [a] Amoxicillin; [b] Norfloxacin; [c] Meropenem.

These activities include antibacterial (Bekhit et al., 2004; Salahuddin et al., 2009; Mohamed et al., 2009; Rostom et al., 2009; Varadaraji et al., 2010; Arulmurugan et al., 2011; Shan et al., 2014; Dai et al., 2015), antifungal (Matysiak et al., 2003; Upadhayaya et al., 2004; Mohite et al., 2011), anti-tuberculosis (Karabanovich et al., 2015; Němeček et al., 2017), antiviral (Miles et al., 1978; Hutchinson & Naylor, 1985), analgesic (Rajasekaran & Thampi, 2004), anti-inflammatory (Pande et al., 1987; Ikeda et al., 1992), anticancer (Bhaskar & Mohite, 2010; Kumar et al., 2011; El-Sayed et al., 2012), hypoglycemic (Momose et al., 2002), and antihypertensive (Sharma et al., 2010), amongst others.

Further, a number of studies have examined the influence of the position of a substituent group in the tetrazole on different types of activities. For example, consistent with the current study, reports depicted the antitubercular potency, selectivity and toxicity of tetrazole derivatives containing different substituents (Karabanovich et al., 2014; Karabanovich et al., 2015; Karabanovich et al., 2016). In these works, the authors clearly determined the influence of the position of substituent groups in the tetrazole upon antimycobacterial activity. For instance, amongst all the compounds produced, 1,5- and 2,5-regioisomer tetrazole derivatives revealed the highest antimycobacterial effect, with minimal inhibitory concentration values of approximately 1 μM (0.37 – 0.46 μg/mL) against a *Mycobacterium tuberculosis* strain (Karabanovich et al., 2015). Furthermore, the 2,5-regioisomers exhibited reproducibly higher antimycobacterial activity compared to the 1,5-isomers. In addition, selectivity and toxicity were evidently affected by the positions in the tetrazole group. More recently, the same research group revealed that the introduction of a tetrazole-5-thioalkyl moiety at position 2 of the tetrazole increased even more the antimycobacterial activity, presenting a stronger *in vitro* activity against *M. tuberculosis* as well as a significant activity against non-tuberculous mycobacterial strains (Němeček et al., 2017).

As mentioned in other sections, Ortar and coworkers (2007) synthesized a series of 18, 1,5- and 2,5-disubstituted carbamoyl tetrazoles, including both LY2183240 regioisomers, and revealed that on inhibition of anandamide cellular uptake, most 1,5-isomers are considerably more potent than the corresponding 2,5-isomers. Still, structurally less flexible substituents in the 5-position appear to yield better inhibitors than more flexible ones. On the other hand, 2,5-isomers can also be more potent against FAAH when compared with their 1,5-equivalent.

On the whole, the position of substituent groups in the tetrazole seems to be a crucial factor for the potency of the molecule. The anti-staphylococcal effect of 2,5-LY2183240 presented in this study is unprecedented in the literature. Many reports showed the activity of these compounds against eukaryotic serine hydrolases, including FAAH and MAGL, related to the putative anandamide membrane transporter (Alexander & Cravatt, 2006; Ortar et al., 2007; Ortar et al., 2008; Maione et al., 2009). However, there are no reports of the activities of these regioisomers towards bacteria.

In this context, the observation that the 2,5-regioisomer has potent anti-staphylococcal activity, warrants further investigation of this isomer as a novel scaffold for the development of treatments of MRSA infections.

Further studies were carried out to establish the mode of action of this specific effect.

4.3.2.2 Effect of Cell Membrane Permeabiliser, PEI, on the Anti-Gram-Negative Activity of 2,5-LY2183240

Gram-negative bacteria possess an outer membrane that acts as a permeability barrier capable of excluding macromolecules or hydrophilic substances, hence the structure is essential for the intrinsic resistance of these microorganisms to antimicrobial agents (Denyer & Maillard, 2002; Nikaido, 2001; Alakomi et al., 2006).

The presence of lipopolysaccharide molecules in the external part of the membrane contributes towards the barrier function of the outer membrane and hence resistance, along with porin type and the presence of multidrug efflux pumps (Nikaido, 2003). Novobiocin, an inhibitor of DNA replication and active against Gram-positive organisms, is ineffective against Gram-negative bacteria like *E. coli* due to the barrier function of the outer membrane.

Despite the fact that the outer membrane guards the cells of Gram-negative bacteria from various external agents, it is possible to modulate this property using different types of agents, commonly known as permeabilizers, which either disrupt the lipopolysaccharide or lipid bilayers or both, increasing the permeability of the outer membrane (Alakomi et al., 2006). For example, polyethyleneimine (PEI), a cationic polymer, has been acknowledged as a permeabilizer that operates by interpolating

into the outer membrane rather than releasing lipopolysaccharides (Helander et al. 1997).

Table 11: Effect of PEI on the susceptibility of *E. coli* 10418 to novobiocin and 2,5-LY2183240 as determined by the broth microdilution method.

	MIC (μg/mL)				
	PEI	Novobiocin	Novobiocin + PEI[a]	2,5-LY	2,5-LY + PEI[b]
***E. coli* 10418**	> 256	256	0.5	> 512	~ 64

[a]MIC of novobiocin in the presence of a fixed concentration of PEI (256 μg/mL).

[b]MIC of 2,5-LY2183240 in the presence of a fixed concentration of PEI (256 μg/mL).

PEI successfully led to the permeation of both novobiocin and 2,5-regioisomer, but to different extents against *E. coli* 10418 (Table 11). Novobiocin had a MIC value of 256 μg/mL, however, in the presence of PEI the MIC reduced more than 500-fold to 0.5 μg/mL. With 2,5-LY2183240, an MIC greater than 512 μg/mL was noted, but in the presence of PEI, despite two-fold variations in the data, a decrease to 64 μg/mL was noted (modal value of five determinations).

These findings mirror those of the previous studies that have examined the effect of PEI and novobiocin towards a *Salmonella typhimurium* strain (Helander et al., 1997; Helander et al., 1998). The authors demonstrated that at concentrations from 20 μg/mL, PEI increased the bacterial uptake of a hydrophobic compound whose activity would be significantly decreased by the outer membrane. Furthermore, through an agar diffusion method, PEI induced susceptibility to novobiocin and other antibiotics towards different Gram-negatives, including *E. coli*. Yet, the antimicrobial agents were not effective in the absence of PEI. Moreover, it is noteworthy that PEI did not inhibit the growth of *E. coli* to any significant extent.

These data indicate that the antimicrobial activity of 2,5-LY2183240 revealed in the previous chapter was selective to Gram-positives potentially due to the presence of the outer membrane and consequently is the basis of intrinsic resistance within Gram-negatives (at least *E. coli*). However, the fact that the 2,5-isomer was effective

against *S. aureus* and not *E. faecalis*, for instance, is still open for discussion and need further studies to enlighten this intriguing effect.

4.3.2.3 In Vitro *Protein Synthesis Inhibition*

Protein synthesis is one of the major targets in the cell for antimicrobial agents (Wilson, 2009). Bacterial ribosomes diverge in structure from their counterparts in eukaryotic cells. Antibacterial agents take advantage of these differences to selectively inhibit bacterial growth (Tenover, 2006). The bacterial ribosome is a cytoplasmic nucleoprotein particle whose main function is to serve as the site of mRNA translation and protein synthesis. The ribosome has a mass of about 2.5 MDa, with RNA accounting for 2/3 of the mass. It consists of two subunits denoted 30S (small subunit) and 50S (large) (Laursen et al., 2005). When joined, the ribosome has a sedimentation coefficient of 70S as opposed to 80S due to tertiary structure. The different subunits are the sites of inhibition for different classes of antimicrobial agents, for example, aminoglycosides, and tetracyclines bind to the 30S subunit of the ribosome, whilst chloramphenicol binds to the 50S subunit (Spector et al., 2002).
During protein synthesis a ribosome moves along on mRNA molecule, reading the codons and adding the correct amino acid (from the corresponding aminoacyl tRNA) to the growing protein. When a stop codon is reached, translation ceases, and the mRNA and protein are released (Weaver, 2012).
In this current study, all the findings so far gathered about the mode of action of the anti-staphylococcal activity of the LY2183240 regioisomers, including the bacteriostatic effect, may suggest potential protein synthesis inhibition.
The *in vitro* protein synthesis experiments were performed using the commercially available cell-free transcription-translation system composed of purified components (PURExpress). The main goal of this assay was to verify if LY2183240 regioisomers could interact or inhibit protein synthesis, especially via ribosomal subunits.
Figure 4.6 shows the SDS-PAGE gel of dihydrofolate reductase (DHFR) protein expression. It was observed that when the DNA was not present in the reaction solution, no DHFR was synthesized (lane 2). Conversely, once the DNA was added to the reaction, the enzyme DHFR, which possesses a size of approximately 20 kDa, was produced (lane 3). Moreover, when LY2183240 regioisomers were supplemented, the presence of the enzyme was noted, indicating no significant

alteration to DHFR synthesis (lanes 4 and 5). As a positive control, chloramphenicol, a standard protein synthesis inhibitor was used; total inhibition of the DHFR synthesis was observed (lane 6).

Protein synthesis is a complex, multi-step process involving many enzymes as well as conformational alignment. The aminoacyl tRNA synthetases that activate each amino acid required for peptide synthesis may by targeted by antibiotics such as mupirocin, which specifically inhibits isoleucine tRNA synthetases in Gram-positive bacteria (e.g. staphylococci and streptococci). However, the majority of antibacterial agents that stop bacterial protein synthesis interfere with the processes involving the 30S or 50S subunits of the 70S bacterial ribosome (Maguire, 2009). The primary steps in the process that are attacked are (i) the formation of the 30S initiation complex, (ii) the formation of the 70S ribosome by the 30S initiation complex and the 50S ribosome, and (iii) the elongation process of assembling amino acids into a polypeptide.

Chloramphenicol is a bacteriostatic agent that prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome (Brock & Brock, 1959; Jardetzky, 1963). It specifically binds to A2451 and A2452 residues in the 23S rRNA of the 50S ribosomal subunit, preventing peptide bond formation (Schifano et al., 2013).

This simple *in vitro* assay could demonstrate the inhibitory activity of chloramphenicol towards the DHFR synthesis, validating the applied method.

Overall, these findings indicate that neither regioisomers have any relevant effect on the synthesis of dihydrofolate reductase.

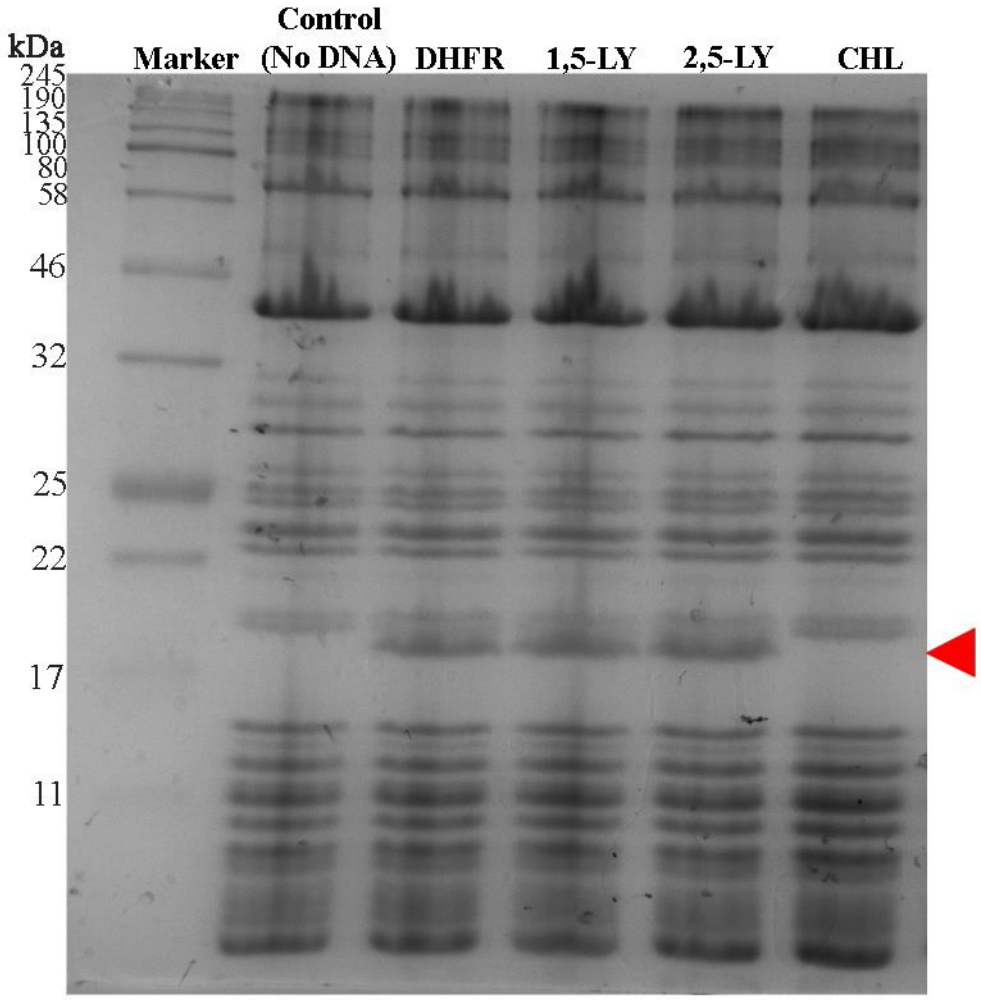


Figure 4.6. Protein expression using the PURExpress™ *in vitro* protein synthesis kit in presence of LY2183240 regioisomers and the standard chloramphenicol (CHL). Around 25 µL reactions containing 250 ng template DNA of DHFR was incubated at 37°C for 2 hours. 2.5 µL of each reaction was analyzed by SDS-PAGE using a 10% Tris-glycine gel. The red arrow indicates the protein of interest, dihydrofolate reductase.

4.3.2.4 Wall Teichoic Acid and Lipoteichoic Acid Biosynthesis Inhibition

Wall teichoic acids are a major component of the Gram-positive bacteria cell wall. They consist of anionic and carbohydrate-based polymers that are covalently attached to the peptidoglycan matrix (Ward 1981; Shockman & Barren 1983; Neuhaus & Baddiley 2003; Meredith et al. 2008). Some of the main functions of the these acids are involved in the determination of cell shape in *S. aureus*, assisting relevant roles in host colonization, virulence, coordination of peptidoglycan synthesis and resistance to β-lactam antibiotics (Sewell & Brown, 2014). More

recently, due to these characteristics, wall teichoic acid biosynthesis has appeared as an authentic new antimicrobial target against *S. aureus*.

The SDS-PAGE analysis of wall teichoic acids isolated from *S. aureus* 12981 grown in increasing but sub-inhibitory concentrations of tunicamycin, a standard inhibitor, shows a dose-dependent reduction in wall teichoic acid biosynthesis (Figure 4.7A).

By contrast, when 2,5-LY2183240 was present, under the same conditions, no significant effect was observed (Figure 4.7B).

These results observed are similar to those of the previous study that have assessed the effect of tunicamycin against wall teichoic acid isolated from *S. aureus* RN4220 (Swoboda et al., 2009). The authors revealed a similar profile for this compound, showing a concentration-dependent decrease in the wall teichoic acid biogenesis. Nevertheless, in this current study a higher concentration of tunicamycin, still below the MIC was used. Many studies have reported the minimum inhibitory concentration of tunicamycin to be around 0.4 μg/mL (Hancock et al., 1976; Pooley & Karamata, 2000; Campbell et al., 2011). In addition, at this concentration, tunicamycin inhibits WTA expression (Atilano et al., 2010; Campbell et al., 2011). On the first attempt, the same concentration range as described in the literature was used (0 – 0.08 μg/mL) (Swoboda et al., 2009). However, no inhibition was detected. After some modifications and endeavours, a significant decrease in the WTA assembly was noticed with tunicamycin (0.16 μg/mL), but not 2,5-LY2183240.

According to the literature, tunicamycin is a selective TarO inhibitor at low concentrations. TarO belongs to a group of essential enzymes that contribute to the synthesis of the wall teichoic acid adding polymers like poly(ribitol-phosphate) and sugars such as *N*-acetylglucosamine (Brown et al., 2013). Hence, it can be used to block WTA expression at concentrations that have no effect on bacterial growth.

The 2,5-regioisomer, albeit using concentrations up to the MIC, had no inhibitory effect, suggesting that the target of the antimicrobial activity was not associated with the WTA biosynthesis pathway. However, further studies are necessary to confirm this possibility.

Besides WTA, another important teichoic acid present in *S. aureus* is lipoteichoic acid (LTA). LTAs are anchored in the bacterial membrane via a glycolipid, whereas WTAs are covalently attached to peptidoglycan (Ward, 1981; Neuhaus & Baddiley, 2003). Lipoteichoic acid can be found in the cell membrane of virtually all Gram-positive bacteria and is liberated into the circulation after antimicrobial treatment.

Moreover, *in vivo*, LTA has been revealed to contribute greatly to the onset of sepsis and septic shock (Alkan & Beachey, 1978; Fischer et al., 1990; Kengatharan et al., 1998; Ginsburg, 2002; Hattar et al., 2006).

Furthermore, this type of teichoic acid synthesis involves the polymerization of polyglycerol-phosphate and its transfer to glucosyl-diacylglycerol (Glc2-DAG) (Koch et al., 1984).

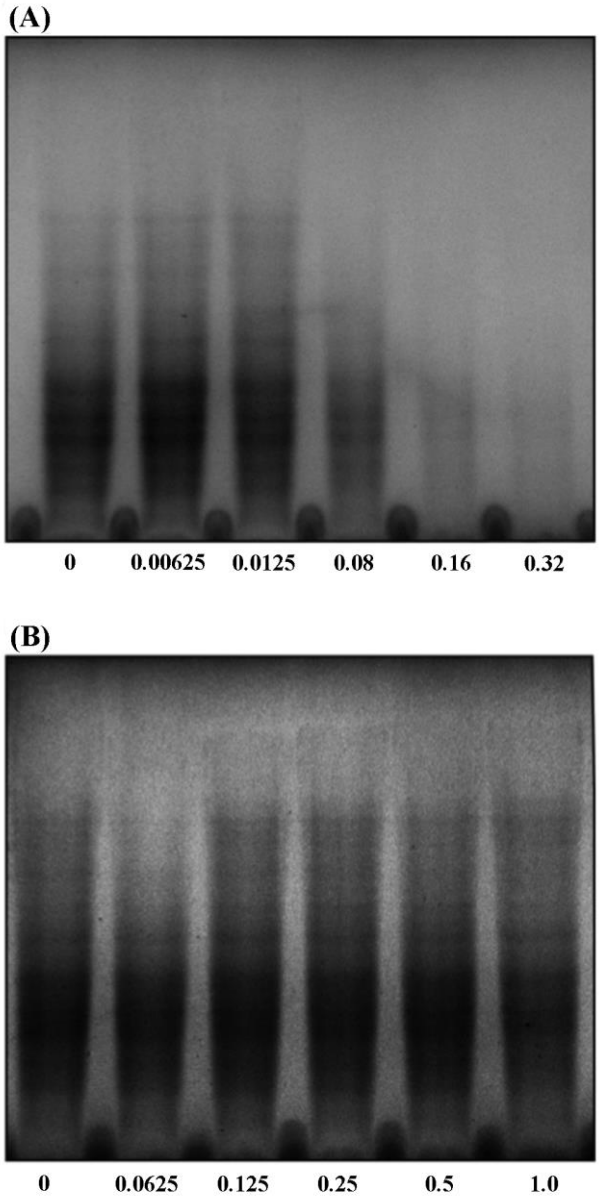


Figure 4.7. PAGE analysis of WTA isolated from *S. aureus* 12981 grown in increasing concentrations (µg/mL) of **(A)** tunicamycin, a TarO inhibitor, and **(B)** 2,5-LY2183240.

Figure 4.8 illustrates the agar-well diffusion test using both LY2183240 regioisomers against the susceptible *S. aureus* 12981. The main objective of this test was to verify the influence of the presence of LTA upon the activity of 2,5-LY2183240.
2,5-LY2183240 caused a significant zone of inhibition (Figure 4.8). The presence of LTA in the agar-well had no effect on regioisomer activity since the same zone of inhibition was observed, which suggested that the mode of action of 2,5-LY may not be related to the interaction of the molecule with lipoteichoic acid.

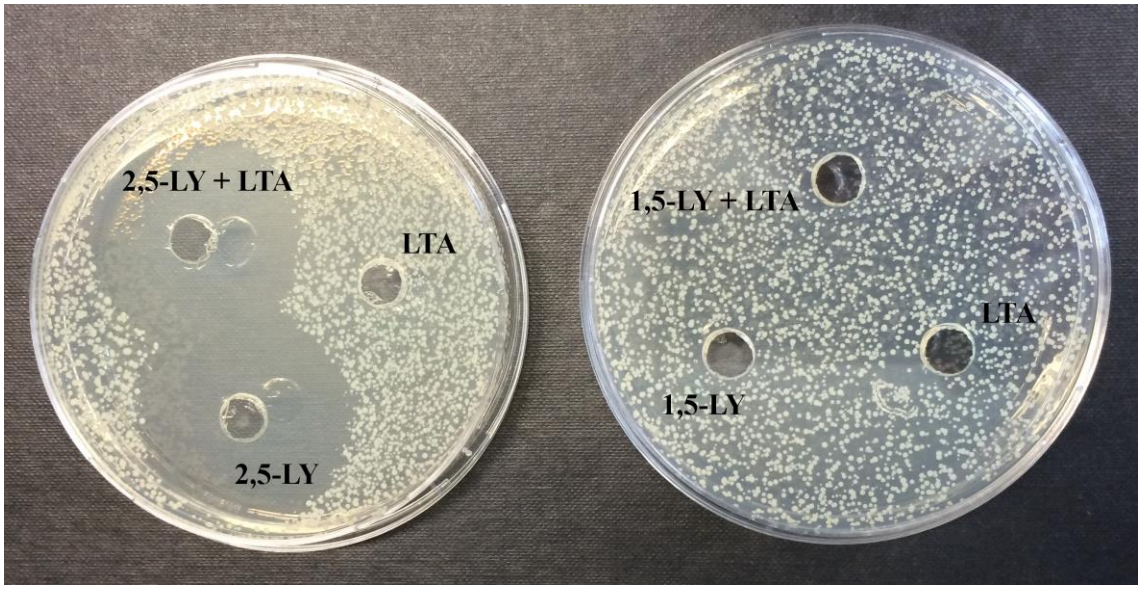


Figure 4.8. Agar-well diffusion test with LY2183240 regioisomers in presence of LTA against *S. aureus* 12981.

In order to explore the potential target or targets of the anti-staphylococcal activity additional studies were performed and discussed in further sections.

4.3.3 Inhibition of Bacterial Fatty Acid Synthesis

Fatty acids play a crucial role in bacterial membrane formation as well as cellular structures and functions (Rock & Jackowski, 2002). Bacteria synthesize fatty acids by utilizing a highly conserved group of enzymes within the type II fatty acid synthase system. Therefore, this system is an attractive target for antibacterial drug discovery (Campbell & Cronan, 2001; Zhang et al., 2003; Heath & Rock, 2004; Yao & Rock, 2016).
Until this point, the data in this study indicate that the potential target or targets of the 2,5-regioisomer could be associated with the membrane of certain Gram-positive

bacteria. A bacteriostatic effect against *S. aureus* was observed, as well as significant effects on the autolysins associated with the peptidoglycan, and a possible link between the murein hydrolases and β-lactamases inhibitory activities. More striking is the spectrum of activity of the 2,5-regioisomer, which mirrors the selectivity observed for fatty acid synthesis inhibitors such as triclosan.

There is a report showing that the inhibition of phospholipid synthesis, accomplished by inhibiting fatty acid synthesis with cerulenin, a FabF and FabB inhibitor, resulted in the simultaneous inhibition of peptidoglycan synthesis (Rodionov & Lshiguro, 1996). Moreover, studies with triclosan, demonstrated that this compound acts as a bacteriostatic agent associated with an inhibition of membrane biogenesis (Gomez Escalada et al., 2005).

Therefore, the main purpose of this section was to assess whether fatty acid synthesis was a potential target or targets of the 2,5-regioisomer thus contributing to the inhibitory activity of the molecule towards *S. aureus*.

A simple assay to test this hypothesis is to supplement the growth media with exogenous fatty acids using for example, Tween 80 or human serum, which are abundant sources of fatty acids. Under these conditions, bacteria would incorporate extracellular fatty acids, circumventing the inhibition of *de novo* fatty acid synthesis and increasing the MIC value of the assessed drug (Brinster et al., 2009; Parsons & Rock, 2011).

In this study triclosan exhibited a potent antimicrobial effect with MIC values of 0.05 μg/mL and 0.5 μg/mL against *S. aureus* and *B. subtilis*, respectively (Table 12). By contrast, in the presence of Tween 80, a significant increase of 80-fold in the MIC towards *S. aureus*, and to a lesser degree, 64-fold against *B. subtilis* was noted. In a similar way, the 2,5-isomer of LY2183240 revealed a potent inhibitory activity against *S. aureus* and to a lesser extent, *B. subtilis*. Nevertheless, in the presence of Tween 80 a dramatic increase in the MIC (greater than 150-fold) towards *S. aureus* was observed. The same increase happened with *B. subtilis*, although in a smaller in magnitude (Table 12). It is worth mentioning that Tween 80 was assessed alone against both organisms and no inhibition of bacterial growth was found.

These findings are consistent with former studies on triclosan. A recent work reported concentration-dependent antimicrobial activity of triclosan against *S. aureus* and *Streptococcus agalactiae*, showing reductions in activities with increasing concentrations of Tween 80 (Krsta et al., 2014). The same study showed a very

similar effect using platensimycin against *S. agalactiae*, but the reverse effect against *S. aureus*. Platensimycin is a selective inhibitor of FabF, an important enzyme in the type II fatty acid synthesis pathway of Gram-positive bacteria (Das et al., 2016).

Table 12. Inhibitory activity of 2,5-LY2183240 in the presence and absence of Tween 80.

	MIC (μg mL^{-1}) of			
Organism	Triclosan		2,5-LY2183240	
	No Tween 80	+ Tween 80 (0.1% v/v)	No Tween 80	+ Tween 80 (0.1% v/v)
S. aureus 12981	0.05	4	2 - 4	> 512
B. subtilis 13	0.5	32	16	64

Bacterial fatty acid biosynthesis is a potentially significant target for antimicrobial treatment, but is not without controversy. As mentioned above, recent reports have suggested that the addition of exogenous fatty acids to growth media may bypass the inhibition of this pathway both *in vitro* and *in vivo* (Brinster et al., 2009). Hence, agents targeting fatty acids synthesis could have restricted *in vivo* relevance for human therapy because fatty acids are highly abundant within the body. However, Balemans et al. (2010) have suggested that the observations of Brinster and co-workers (2009) do not hold true for *S. aureus*. They revealed that human serum had no influence on *fab* gene expression in *S. aureus*, although downregulation of *fab* genes expression for *S. agalactiae* was observed. In a reply to Balemans and coworkers, Brinster concluded that FASII-directed antimicrobials can indeed reach their targets when exogenous fatty acids are present, to inhibit fatty-acid synthesis in *S. aureus*. Nonetheless, FASII inhibition is compensated by the exogenous fatty acids, and allows bacterial growth. Another report also demonstrated that the activity of triclosan *in vitro* is severely compromised by the addition of oleic acid and Tween 80 (Prosen et al., 2011).

Using animal models, other studies on *Staphylococcus* and *Neisseria* species, have shown that exogenous fatty acids are not able to circumvent FabI inhibition in these specific microorganisms (Parsons & Rock, 2011; Yao & Rock, 2015).

Despite this controversy, major pharmaceutical companies are still investigating the potential use of FAS inhibitors as antimicrobial agents, such as platensimycin and platencin (inhibitor of FabF/H). Each has activity against several Gram-positive pathogens, including MRSAs.

The fatty acids present in Tween 80, as shown in this study, are able to compromise the activities of triclosan and 2,5-LY2183240 suggesting a target of 2,5-LY2183240 may lie within the type II fatty acid synthase system.

4.3.3.1 Characterization of 2,5-LY2183240-Resistant Mutants

One of the last steps in the fatty acid synthesis pathway is executed by the enzyme enoyl-acyl carrier protein (ACP) reductase (FabI) (Heath & Rock, 1995). Preceding genomics studies, FabI was assumed to be the only enoyl-ACP reductase in bacteria. Nevertheless, investigation of key bacterial genomes demonstrated that FabI is not present in some micro-organisms, and an alternative enoyl-ACP reductase, FabK, is found instead (Rock & Heath, 2000; Payne et al., 2002). In some cases, both FabI and FabK may occur in the same organism; for instance, FabK is the only enoyl-ACP reductase in *S. pneumoniae* and both FabI and FabK have been discovered in pathogens such as *E. faecalis* and *P. aeruginosa*. Therefore, FabI represents a selective antibacterial target for those pathogens such as *S. aureus*, where FabI is the only enoyl-ACP reductase. Conversely, a compound that possesses inhibitory potency against both FabK and FabI would be expected to possess a far broader spectrum of antibacterial activity. In this context, it is believed that a target of 2,5-LY2183240 could potentially be FabI. Moreover, besides *S. aureus* strains, 2,5-LY2183240 had antimicrobial activity against *B. subtilis* (Table 10). It is recognized that the closest homologies with the FabI structure of *S. aureus* are from the Gram-positive bacteria *Bacillus anthracis* and *B. subtilis*, suggesting that FabI might be a potential target of 2,5-LY2183240 for these organisms too.

A vital part in the discovery and development of antibacterial drugs is a precise evaluation of the mode of action (Cunningham et al., 2013). A major bottleneck in the drug discovery process is the target identification (Silver, 2011).

In general, no systematic target-identification procedures of broad efficacy currently exist. Undeniably, target identification has been described as the ‘missing link’ in

chemical genetics, a technical impediment that restricts the immense potential of the method (Burdine & Kodadek, 2004; Galloway et al., 2009).

There are different strategies described in the literature to identify the potential targets of novel antimicrobial agents. One of the classic approaches is the use of resistant mutants to determine the mechanism of action (O'Neill & Chopra, 2004).

The selection of triclosan- and 2,5-LY2183240-resistant bacterial mutants is illustrated in Figure 4.9. Colonies that have grown in the zone of inhibition were selected and subcultured on agar plates and the MIC of 2,5-LY2183240 determined by a microbroth dilution procedure to quantify the change in susceptibility (if any).

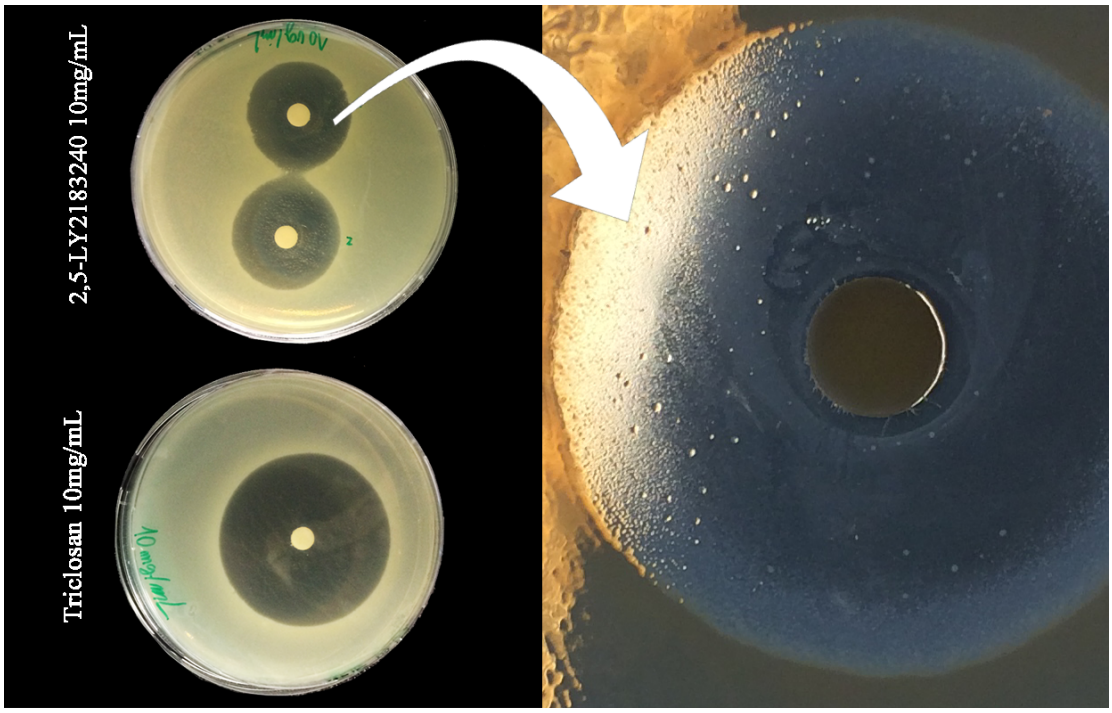


Figure 4.9 Generation of drug-resistant mutants of *S. aureus* 12981.

Antibiotic resistance can be achieved by different modes, such as horizontal acquisition of resistance genes, recombination of foreign DNA into the chromosome, or by mutations in different chromosomal loci (Davies, 1997). Mutation rate is often defined as the *in vitro* frequency at which detectable mutants appear in a bacterial population in the presence of a specific antimicrobial agent concentration. It is noteworthy that in this particular case it is the number of mutant cells that are recorded and not the number of mutation events (Martinez & Baquero, 2000). Consequently, it is only taking into account selectively favorable mutations that led to a visible antibiotic resistance phenotype.

Generally, the determination of the spontaneous mutation frequency at a particular drug concentration is achieved by counting the confirmed resistant-colonies growing on drug-containing plates and dividing by the total inoculum size across all of the plates at that concentration. Although this determination is widely considered an important assignment, at this stage of the project, the mutation frequency was not determined due the availability and price of 2,5-LY2183240; it would be necessary to use significant quantities of the drug in order to determine the mutation frequency.
Examination of the isolates that grew in the inhibition zone, yielded only one isolate (D) that exhibited a significant increase of around 8 to 16-fold in the MIC of 2,5-LY2183240 for the mutant compared to the susceptible wild type parental strain (A) (Table 13). Amongst the isolates selected, no resistance to triclosan was noticed. Curiously, no concomitant reduction in susceptibility to triclosan was observed for isolate D.

Table 13. Minimum inhibitory concentrations of triclosan and 2,5-LY2183240 against suspected mutants of *S. aureus*.

Isolates	MIC (μg/mL)	
	Triclosan	2,5-LY2183240
S. aureus 12981 (A)	0.03	4
S. aureus B	0.03	8
S. aureus C	0.03	4
S. aureus D	0.03	32 – 64
S. aureus E	0.03	4

In order to determine the nature of the reduced susceptibility to the drug, the *fabI* genes and flanking regions from spontaneous 2,5-LY2183240-resistant *S. aureus* isolate (D) and the parental strain (*S. aureus* MSSA 12981; A) were sequenced.
Analysis showed that the sequence of the *fabI* gene and flanking regions, which includes the promoter, were identical (Figure 4.10). Consequently, alterations to the FabI primary sequence were not responsible for the reduced susceptibility observed.

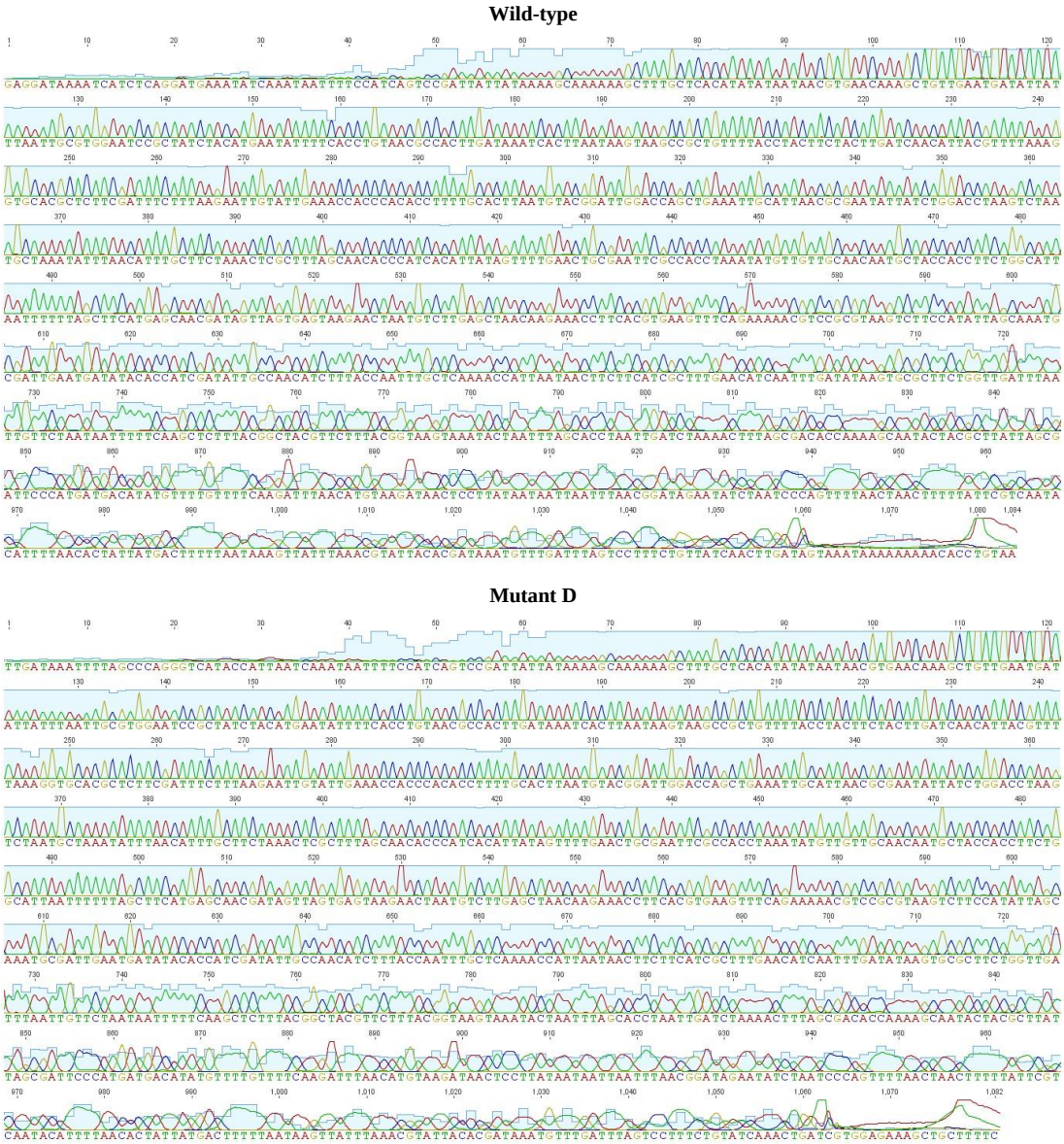


Figure 4.10. Examples of two of the DNA sequencing traces of *fabI* from *S. aureus* (wild type) and 2,5-LY2183240-resistant mutant. Sequence was generated from the reverse-stand primer.

There are several possible explanations for these results; the mutation giving rise to resistance could affect accumulation of the drug in the cell, a metabolic bypass pathway or process could be activated, FabI expression could be affected by an alternative mechanism or FabI is not the main target responsible for antimicrobial activity. Firstly, as mentioned before, a common mechanism of drug resistance is the active extrusion of the antimicrobial agents by efflux pumps (Webber & Piddock, 2003). These pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds. In this case, it could be specific for 2,5-LY2183240 and not triclosan, since isolate D was susceptible to triclosan. It is well

documented in the literature that efflux pumps play an important role in antimicrobial resistance. Mutations in the local repressor gene, or in a global regulatory gene, and also in the promoter region of the transporter gene are some of the mechanisms that lead to increased expression of efflux pumps in clinical isolates (Piddock 2006; Piddock 2006; Li & Nikaido 2004; Sun et al. 2014).

Another possibility might be that the *fabI* mutation is unstable and susceptible to reversion with subsequent loss of the antimicrobial resistance. Hence subculture on drug-free media may have promoted reversion prior to *fabI* PCR amplification and sequence analysis. Resistance mutations may be expected to give rise to a fitness cost since they target important biological functions in the cell. Resistance to antibiotics frequently reduces the fitness of bacteria in the absence of antibiotics, and this is frequently referred to as the "cost" of resistance (Spratt, 1996; Sander et al., 2002). In other words, the acquisition of drug resistance may impose a cost on bacteria affecting such processes as growth rate, generation time and virulence, especially with the first mutation (Johnson et al., 2004; Pope et al., 2010). This cost plays a crucial role in the dynamics of resistance by generating selection against resistance when bacteria encounter an antibiotic-free environment (Vogwill & Maclean, 2015).

As modest increases in the level of FabI protein expression have been correlated with a reduction in sensitivity to FabI inhibitors, and FabI expression can be affected by events independent of promoter modifications, FabI expression for the parental strain and 2,5-LY2183240-resistant mutant were determined by western blotting and anti-FabI antibody (Fan et al., 2002) (Figure 4.11).

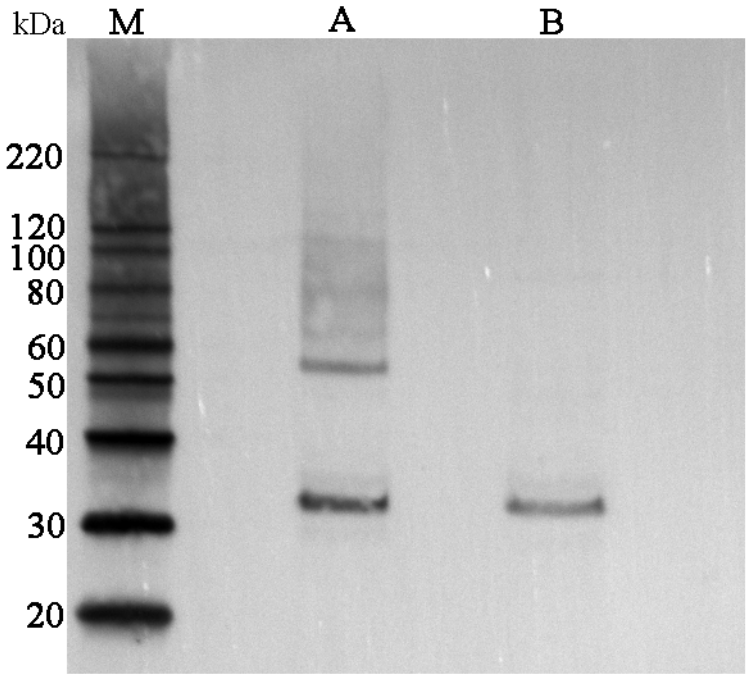


Figure 4.11. Western blot of *S. aureus* 12981 (wild-type) (A), and 2,5-LY2183240-resistant mutant (B) FabI expression. M stands for protein marker.

A band around 30 kDa, that corresponds to FabI protein as previously described in literature was observed in both samples (Ji et al., 2004; Yao et al., 2016). There was no significant increase in FabI expression in the 2,5-LY2183240-resistant isolate when compared to wild-type strain (Figure 4.11B). Actually, the band in sample B seems slightly less concentrated. A lack of over-expression of FabI is consistent with the triclosan susceptibility observed for mutant D; a strain of *S. aureus* overexpressing FabI has been shown by others to give rise to an increase in triclosan MIC, whilst susceptibilities to a range of unrelated antimicrobial agents are unaffected (Slater-Radosti et al., 2001).

Further inspection of the western blot reveals an additional band of 50 to 60 kDa in the profile of the wild-type parental strain. This higher molecular weight band could be due to technical artifacts, for example, the target protein may form multimers, or different quantities of proteins may have been loaded on the gel despite standardisation. To investigate this, an SDS-PAGE gel of both protein extracts was run in parallel with the samples to be blotted and stained with coomassie blue (Figure 4.12). Surprisingly, whilst similar total quantities of proteins appear to have been loaded on to the gel, several differences were noted in the protein profiles between the mutant and parental strain. This is unusual for a single-step mutant and could

indicate variations in protein extraction between samples or a mutation that has affected protein expression globally within the cell.

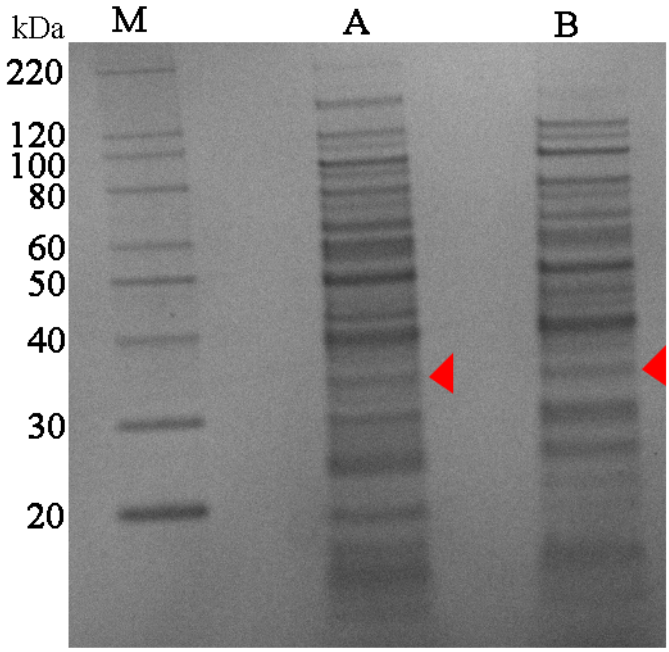


Figure 4.12. SDS-PAGE gel of the protein extracts from *S. aureus* 12981 (wild-type) (A) and 2,5-LY2183240-resistant mutant (B). M stands for protein marker. The gel was stained with coomassie blue. Arrows indicate putative FabI protein bands.

In summary, the antimicrobial spectrum of activity and the capacity of Tween 80 to reduce the potency of 2,5-LY2183240 suggested that the fatty synthesis pathway, particularly the step mediated by FabI was a probable target responsible for the antimicrobial activity of the compound. However, analysis of a 2,5-LY2183240-resistant mutant revealed no significant difference in sequence of the *fabI* gene or in FabI expression. Multiple changes to the protein profile were found for the mutant although the significance has yet to be determined. Whilst no changes to FabI were noted, this enzyme cannot be rule it out at this stage nor can fatty acid synthesis as the putative target. Studies are ongoing to determine the antimicrobial mechanism of action of the 2,5-LY2183240 regioisomer.

4.3.4 Effect of LY2183240 Regioisomers Against β-Lactamases

Chapter 3 presented initial findings of the activities of LY2183240 against class C β-lactamases. Albeit not a pure compound, the LY2183240 mixture had a potent effect

towards AmpC β-lactamases from *E. coli* and *C. freundii*, but lacked significant activity against the class A β-lactamase TEM-1.

In this section the hypothesis that both LY2183240 regioisomers exhibit selective activity towards β-lactamases was assessed with enzymes from class A (TEM-1), class B (metallo-β-lactamase NDM-1), and in order to confirm the activity towards class C β-lactamases, a purified cephalosporinase from *Enterobacter cloacae* sp. was investigated.

4.3.4.1 Class A β-Lactamase TEM-1

Subsequent to the finding that the inhibitory activity of LY2183240, as a mixture, had no significant effect towards class A β-lactamase, both LY2183240 regioisomers were tested independently against TEM-1 with nitrocefin as a substrate.

A significant and progressive increase in the absorbance at 486 nm was observed when 100 μM of nitrocefin was added to the solution containing the enzyme TEM-1 (Figure 4.13). The solvent used to dissolve the samples (DMSO) showed no relevant effect on enzyme activity. However, in the presence of the 2,5-regioisomer (at 420 μM) a significant reduction in enzyme activity was noted (Figure 4.13). After 3 minutes of incubation, the isomer gave rise to a decrease of almost 50 % when compared to the absorbance in the absence of the inhibitor. Likewise, albeit to a lesser extent, the 1,5-isomer led to reduced TEM-1 activity of about 25 % compared to the control. For comparison and method validation determinations, clavulanic acid was used at a concentration of 10 μM and, as anticipated, revealed a strong decrease in the class A β-lactamase action, displaying a 75 % loss in TEM-1 activity after 180 seconds of incubation.

These findings suggest that the position of the carbamoyl in the tetrazole of LY2183240 has a direct impact on inhibitory activity, an effect seen before in this study.

Previously in section 3.7.11, the LY2183240 mixture of both isomers plus other impurities, showed no significant activity against TEM-1. When assessed separately, the 1,5-regioisomer revealed a higher effect towards the same enzyme suggesting that the quantity and concentration of this isomer was crucial for the inhibitory activity.

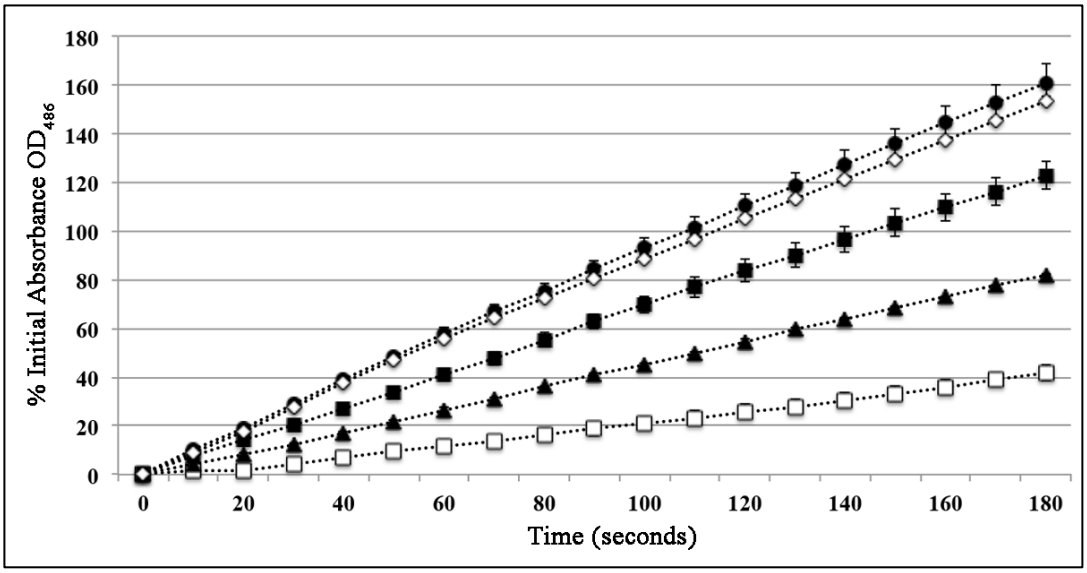


Figure 4.13. The activity of class A β-lactamase TEM-1 (penicillinase) using nitrocefin as a substrate (●, control) and in presence of 10 μM of clavulanic acid (□), 420 μM 1,5-LY2183240 (▲) or 420 μM 2,5-LY2183240 (■). DSMO was used as a solvent control (◇).

This more potent effect of 1,5-isomer seems to be in agreement with earlier findings of LY2183240 activities (Moore et al., 2005; Alexander & Cravatt, 2006; Ortar et al., 2007). These studies revealed that 1,5-LY2183240 demonstrated higher pharmacological potency towards serine hydrolases from eukaryotic cells than 2,5-LY2183240.

Throughout this thesis it was possible to observe that the two regioisomers presented different potencies and activities depending on the type of enzyme investigated.

It should be stressed that although an inhibitory effect against the class A penicillinases TEM-1 was observed, a larger quantity of the drug was used (420 μM) when compared to clavulanic acid (10 μM). Furthermore, this inhibitory activity is substantially lower when compared with the activities of the regioisomers against class C enzymes. This aspect will be explored and discussed in detail within section 4.3.4.7 of this Chapter.

4.3.4.2 Class B β-Lactamase (Metallo-β-Lactamase)

The New Delhi metallo-β-lactamase 1 (NDM-1) was first acknowledged in 2008 within *Klebsiella pneumoniae* isolated from a Swedish patient relocated from India. This β-lactamase is described as a carbapenemase that hydrolyzes all β-lactams excluding monobactams, being sensitive to EDTA but not to clavulanic acid

(Nordmann et al., 2011; Arpin et al., 2012). This enzyme is encoded by the gene bla_{NDM-1} and it is generally carried by conjugative plasmids that possess multiple additional determinants, contributing towards the multidrug resistance phenotype (Göttig et al., 2010).

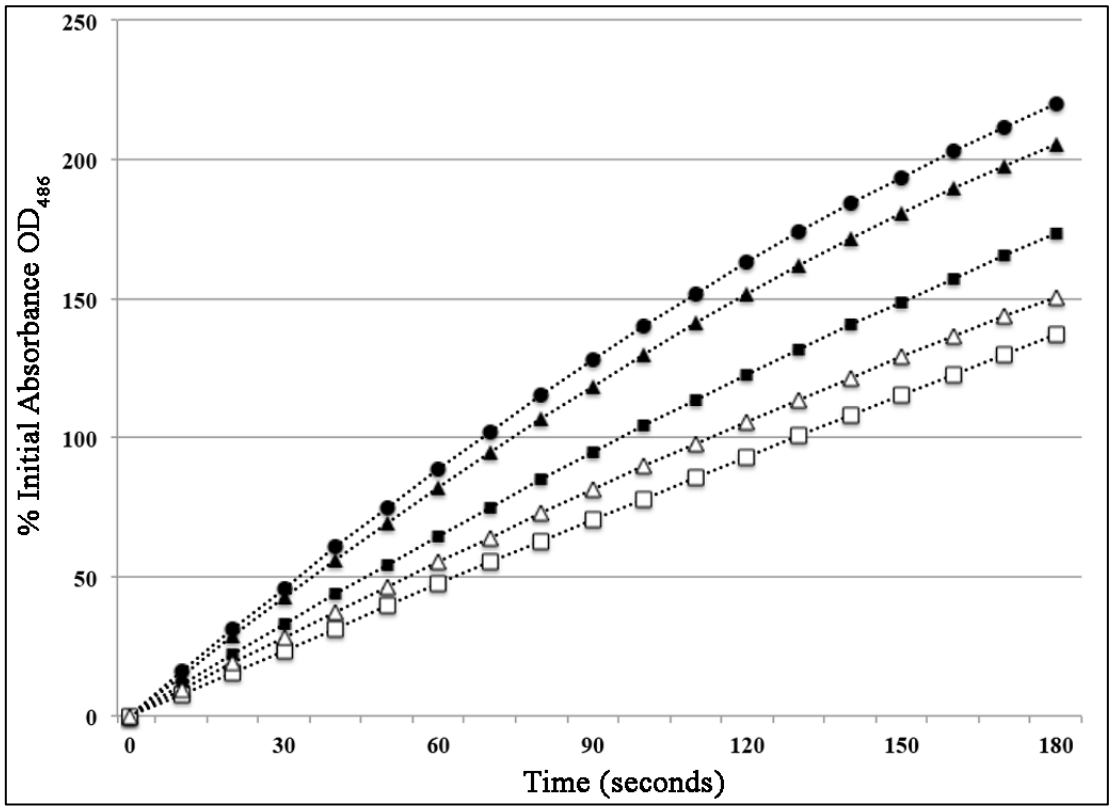


Figure 4.14. Progressive curves with different concentrations of the LY2183240 regioisomers; 1,5-LY2183240 at 420 μM (■), 42 μM (▲) and 2,5-LY2183240 at 420 μM (□), 42 μM (△), against supernatant from *Klebsiella pneumoniae* containing the class B β-lactamase NDM-1 (●, control) and the substrate nitrocefin (100 μM). For some points, the error bars would be shorter than the height of the symbol. In these cases, the software GraphPad Prism does not draw the error bars.

In this current study, for comparison purposes, the supernatant of *Klebsiella pneumoniae* NCTC 13443 containing the metallo-β-lactamase NDM-1 was used. The results revealed a significant increase of around 220 % of the initial absorbance at 486 nm when incubated with the substrate nitrocefin (Figure 4.14), indicating the presence of the metallo-β-lactamase. In the presence of LY2183240 regioisomers, a gradual and concentration-dependent reduction in the enzyme activity was observed. Surprisingly, the 2,5-isomer revealed to be more potent than 1,5-LY2183240 showing a significant decrease at both concentrations tested. At a concentration of 420 μM for instance, the 2,5-isomer exhibited a decrease of about 35 % after 3

minutes incubation. At the same concentration, the isomer 1,5 showed a similar inhibitory effect, although to a lesser extent, giving rise to a 20 % reduction in the NDM-1 activity. Again, the results suggest that the position of carbamoyl in the tetrazole of the molecule influenced directly the inhibitory activity against NDM-1.

From a chemical point of view these findings seem to be in agreement with previous studies about the position of the substituent group in the tetrazole. Compounds with a similar structure to LY2183240, i.e., possessing biphenyl tetrazoles, demonstrate significant activity towards metallo-β-lactamases (Toney et al., 1998; Toney et al., 1999). Toney and co-workers screened a Merck chemical collection of biphenyl tetrazoles linked to various heterocyclic aromatic rings. The study revealed a potent and competitive inhibitory effect against a metallo-β-lactamase from *Bacteroides fragilis* as well as good activity when used in combination with imipenem. Intriguingly, it was demonstrated that the position relative to the biphenyl ring system in the tetrazole group influenced the activity. For instance, the *ortho* position was discovered to be essential for enzyme inhibition; movement of the tetrazole group to the *meta* or *para* positions resulted in IC_{50} values of 10 to 20 mM (Toney et al., 1998). In addition, replacement of carboxamide for the tetrazole group at the *ortho* position also led to a reduction of the inhibitory effect. Comparably, movement of the carboxamide group to the *meta* or *para* positions also led to a loss of activity with IC_{50} values between 2.5 and 1.0 mM, respectively.

Since its identification, this class B β-lactamase has become a challenging menace to public health, encouraging the World Health Organization to issue a global warning (Feng et al., 2014). Moreover, as mentioned in Chapter 1, the hydrolytic mode of metallo-β-lactamases are in general considerably distinctive comparing to the other classes, requiring one or two atoms of zinc (Palzkill, 2013; Drawz et al., 2014). The substrate profile comprises all known β-lactams and β-lactamase inhibitors, except monobactams such as aztreonam, illustrating a highly extensive substrate profile.

In this regard, this class of β-lactamases represents a particular challenge for clinicians and researchers of the field. However, to illustrate some of the achievements, a series of mercaptoacetic acid thiol ester derivatives have been identified as potent metallo-β-lactamase inhibitors and initiated further investigation of thiols as potential lead compounds (Payne et al., 1997). *L*-Captopril, for instance, used to treat hypertension by angiotensin-converting enzyme inhibition, has been reported to inhibit metallo-β-lactamase by chelating to the catalytic site zinc ions

through its thiolate (Li et al., 2014; Brem et al., 2015). The authors revealed correlations between the binding modes and inhibition potency using high-resolution crystal structures of three metallo-β-lactamases (IMP-1, BcII and VIM-2) in complex with both *L*- and *D*-captopril stereoisomers. Consecutively, other compounds containing the sulfur atom, such as thiol carboxylates (Concha et al., 2000), as well as thiols (Siemann et al., 2003; Liénard et al., 2008) and thiomandelic acid (Mollard et al., 2001) were assessed and revealed significant effect (Faridoon et al., 2012; Yang et al., 2015; Feng et al., 2014; Zhang et al., 2014). More recently, triazolylthioacetamides compounds demonstrated specific inhibitory effect against β-lactamase NDM-1 with an IC_{50} down to 1.90 μM, however, no activity against other metallo-β-lactamases was noted (Zhai et al., 2016).

Although interesting activity was demonstrated, none of the promising inhibitors mentioned have significant prospect against class B enzymes (Drawz et al., 2014).

In this study evaluation of the MIC values of LY2183240 regioisomers towards *K. pneumoniae* expressing NDM-1 as well as *K. pneumoniae* 17 expressing a CTX-M-type β-lactamase revealed no activities against these particular microorganisms (Table 14). Similarly, as demonstrated in the previous section, this result suggests that both isomers exhibit a specific activity against the serine hydrolase (β-lactamase), but not the microorganism per se. Still, it was possible to verify the resistance of the *K. pneumoniae* NDM-1-producing strain with meropenem as a reference. Meropenem is a broad-spectrum antibacterial agent of the carbapenem family, suggested as empirical therapy preceding to the identification of causative microorganisms, or for a disease caused by single or multiple susceptible bacteria in patients with a broad range of serious infections (Baldwin et al., 2008). In previous reports, this antimicrobial agent revealed good *in vitro* activity against clinically relevant Enterobacteriaceae, including *C. freundii*, *E. cloaceae*, *E. coli* and *K. pneumoniae*. In this current study, meropenem exhibited MIC values of 8 to 16 μg/mL against *K. pneumoniae* 17 and higher than 128 μg/mL for the NDM-1-producing strain. These results substantiate previous findings in the literature. Worthington et al. (2012) demonstrated a similar MIC value of meropenem against the NDM-1-producing strain (256 μg/mL). In regards to *K. pneumoniae* 17, meropenem showed a potent effect, however with a MIC value higher than presented in literature for a susceptible organism (Baldwin et al., 2008).

Table 14. MICs of LY2183240 regioisomers and meropenem against *K. pneumoniae*.

	MIC (μg/mL)		
Organism	Meropenem	1,5-LY2183240	2,5-LY2183240
K. pneumoniae NDM-1	> 128	> 128	> 128
K. pneumoniae 17	8 − 16	> 128	> 128

It is worth mentioning that, as seen in the Chapter 3, the LY2183240 mixture presented a significant effect against class C β-lactamases from *E. coli* G69 and *C. freundii* 382010, and no significant activity towards the class A enzyme TEM-1. In this sense, the main motivation to test LY2183240 isomers against metallo-β-lactamases is to verify the specificity of these compounds towards different classes of β-lactamases. The activity of both regioisomers found in this section against NDM-1 albeit intriguing, is much less potent when compared to AmpC β-lactamases. Hence, the following sections of Chapter 4 focus on the activity towards class C of these enzymes.

4.3.4.3 Purification of the Class C β-Lactamase from Enterobacter cloacae

Innumerable proteins have been purified in active forms based on features such as size, solubility, charge, or specific binding affinity. Commonly, protein extracts or mixtures are subjected to a sequence of separations, which are based on the differentiating properties of the target with an to aim to obtain a pure protein (Berg et al., 2002).

The literature describes a diversity of methods used to purify cephalosporinases from *Enterobacter cloacae* (Minami et al., 1980; Charlier et al., 1983; Cartwright et al., 1984; Graham & Mantle, 1989). With respect to class C β-lactamases, it is well recognized that boric and boronic acids act as reversible competitive inhibitors of this enzyme type (Beesley et al., 1983; Crompton et al., 1988). Thereby, many β-lactamases from class C have been purified by affinity chromatography utilising boronic acid-containing gels. The process is selective, efficient and fast, allowing

one-step purification of large quantities of enzyme from crude protein extracts (Cartwright et al., 1984).

Figure 4.15 is an example of SDS-PAGE analysis of the β-lactamase from *Enterobacter cloacae* in a sample with a composition of around 60 % protein (0.2-0.6 units/mg protein) purchased from Sigma-Aldrich, UK.

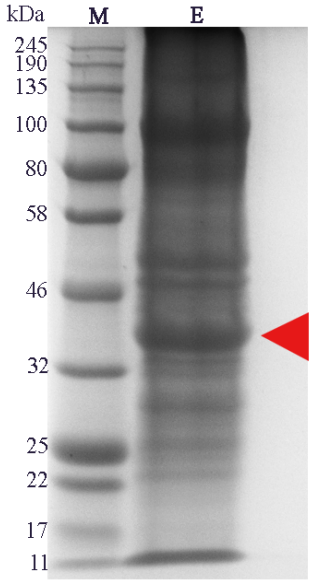


Figure 4.15. SDS-PAGE (10 % gel) of sample (100 µg) containing the β-lactamase from *Enterobacter cloacae*. M, molecular weight marker, E, extract of β-lactamase from *Enterobacter cloacae*. The red arrow shows the position of class C enzyme in the gel.

Analysis of the sample revealed the presence of several bands from 11 to 245 kDa, including a ~39 kDa band that corresponds to the AmpC β-lactamase.

A 100 mg-sample of this protein mixture was subjected to purification by affinity chromatography on phenylboronic acid-agarose according to the method developed by Cartright and Waley (1984). After the dialysis phase, the crude protein was eluted with 0.5 M-borate/0.5 M-NaCl (pH 7) and fractions of approximately 5 mL each were collected throughout the procedure. β-Lactam hydrolytic activities of the fractions were monitored during the purification process with nitrocefin as the chromogenic substrate in order to verify the presence of the β-lactamase in the fractions.

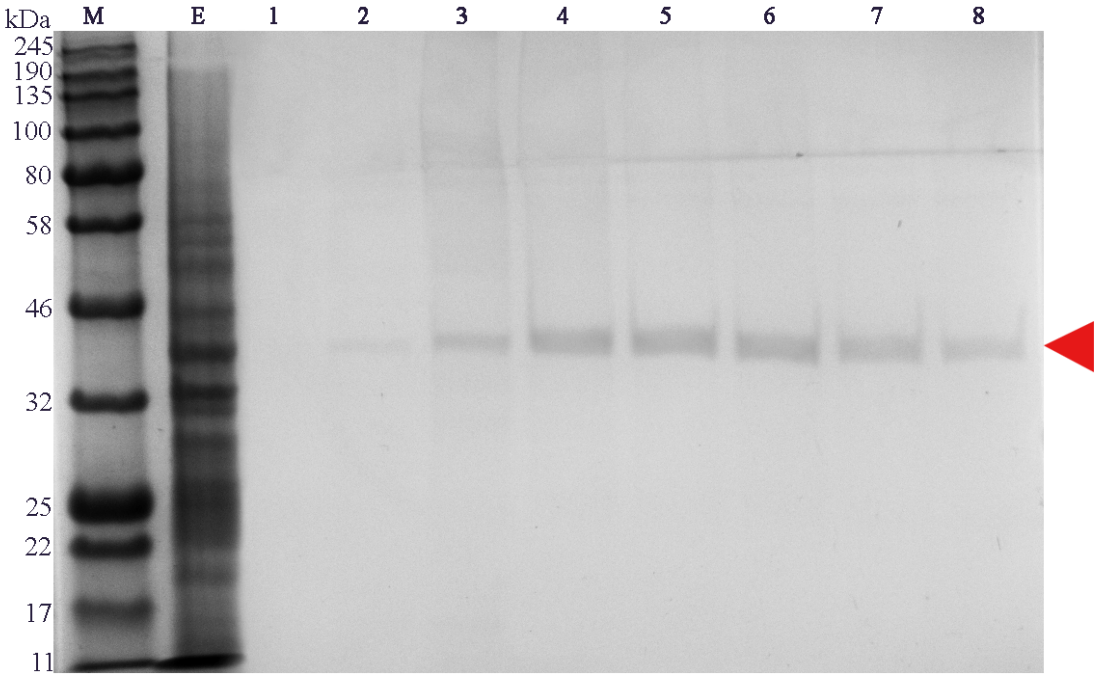


Figure 4.16. SDS-PAGE (10% gel) of fraction obtained during the purification process utilising a boronic acid column. M, molecular weight marker; E, extract of β-lactamase from *Enterobacter cloacae* P99; lane 1, Fraction 1; lane 2, Fraction 2; lane 3, Fraction 3; lane 4, Fraction 4; lane 5, Fraction 5; lane 6, Fraction 6; lane 7, Fraction 7; lane 8, Fraction 8; The red arrow shows position of P99 enzyme in the gel.

As judged by SDS–PAGE (Figure 4.16), the AmpC β-lactamase started to elute in quantity from fraction 3 to fraction 8, yielding a single band of around 39 kDa. The collected fractions containing AmpC β-lactamase were then combined and concentrated by means of a spin-filter with 10 kDa molecular weight cutoff (Figure 4.17).

The results were consistent with those of other researchers who have purified β-lactamases with the same technique (Cartwright et al., 1984; Marchou et al., 1987; Stachyra et al., 2010). Although a significant degree of purification had been achieved by the single step of purification process using boronic affinity chromatography, contaminating proteins were still present. Consequently, it was necessary to subject the sample to a further round of purification this time employing Fast Protein Liquid Chromatography utilizing a HiLoad 16/60 Superdex 75 column (GE Health) and β-lactamase elution with KH_2PO_4 (pH 5.5).

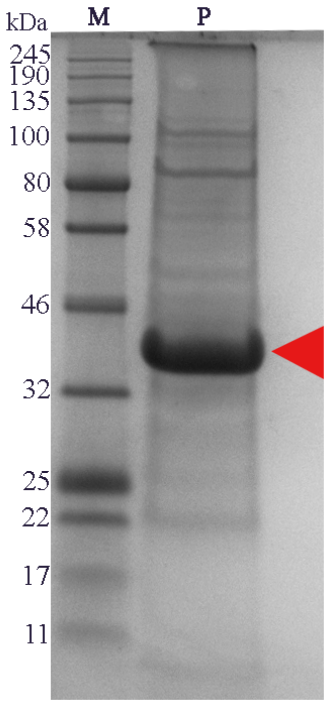


Figure 4.17. SDS-PAGE (10 % gel) of the β-lactamase from *Enterobacter cloacae* after purification and desalting. **M**, molecular weight marker, **P**, purified extract of β-lactamase from *Enterobacter cloacae* sp. The red arrow shows the position of the β-lactamase in the gel.

Figure 4.18 shows the SDS-PAGE result of the purification process with FPLC. As expected, after the procedure, the purified soluble β-lactamase was present with a high degree of purity, illustrated by a single and clear band with a molecular mass approximately of 39 kDa in all the fractions collected.

The fractions containing the β-lactamase (fractions 4 to 10) were combined and concentrated as previously described. The other fractions containing contaminants or other proteins were discarded (data not shown).

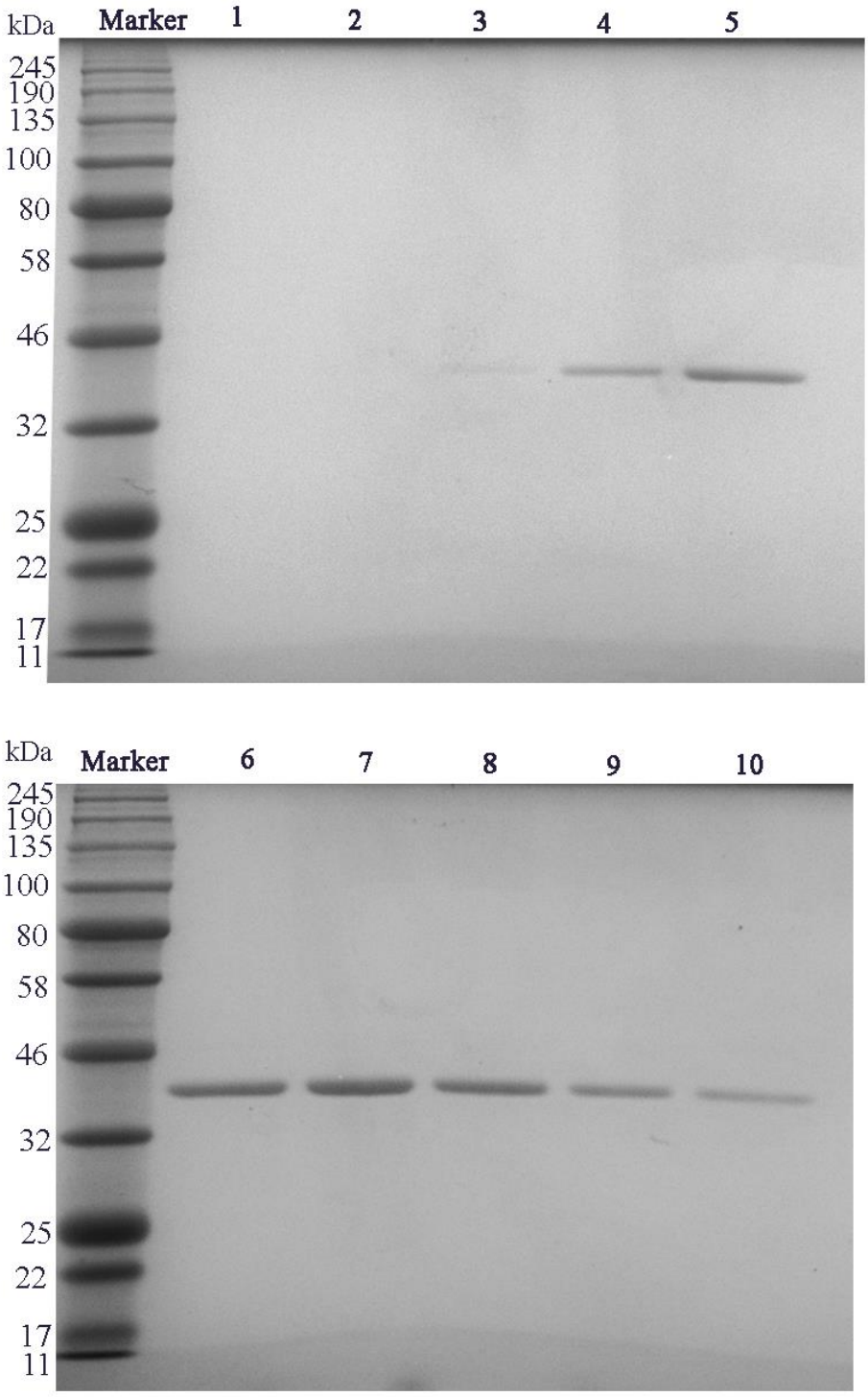


Figure 4.18. SDS-PAGE (10 % gel) of the β-lactamase from *Enterobacter cloacae* after FPLC utilizing a Superdex 75 column. M, molecular size marker; the number represent the fractions collected in the order obtained.

The purified enzyme was then quantified by the Bradford method (Figure 4.19).

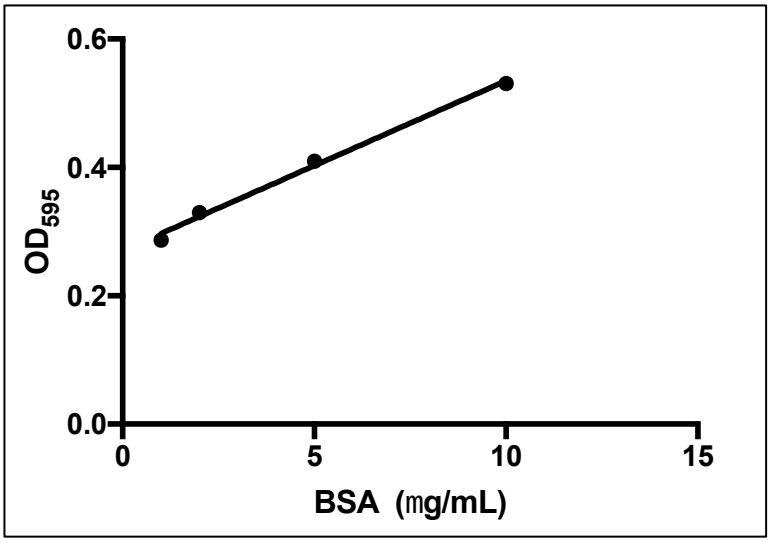


Figure 4.19. BSA calibration curve using the Bradford reagent. The BSA standard curve with a concentration range of 2 – 15 µg/mL is shown. The measurements were performed in triplicate. For some points, the error bars would be shorter than the height of the symbol and consequently these are not drawn.

$$\mathbf{Y = 0.02645x + 0.2705}$$

$$\mathbf{R^2 = 0.99388}$$

The results show that boronic acid agarose gels together with FPLC using a Superdex 75 column provided a reasonable and effective method for purifying AmpC β-lactamase from the protein mixture. The purified cephalosporinase was then used in different types of experiments, including a steady-state kinetic assay.

4.3.4.4 Effect of URB597 against class C β-lactamase

URB597 (cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester), previously know as KDS-4103, is a crystalline white solid with a molecular weight of 338.4 (Piomelli et al., 2006). The compound has two hydrogen bond donors and five hydrogen bond acceptors (Figure 4.20).

Several studies have defined URB597 as a strong FAAH inhibitor *in vitro* and *in vivo* (Kathuria et al., 2002; McKinney & Cravatt, 2005; Alexander & Cravatt, 2006; Dickason-Chesterfield et al., 2006; Petrosino et al., 2009; Powers et al., 2010; Mileni et al., 2010). This compound is reported to bind in the hydrophobic site and catalytic

pocket of FAAH that connects the active site residues to the membrane surface of FAAH (Mor et al., 2004).

LY2183240, like the FAAH inhibitor URB597, potently inhibits FAAH activity by carbamylation of the enzyme's serine nucleophile. Nevertheless, unlike URB597, LY2183240 is not selective for FAAH as multiple other serine hydrolases are inactivated by LY2183240, according to several studies (Alexander & Cravatt, 2006; Ortar et al., 2007; Ortar et al., 2008).

The compound URB597 was assessed in order to verify if there is a relation between FAAH inhibitory effect and the activity against β-lactamases.

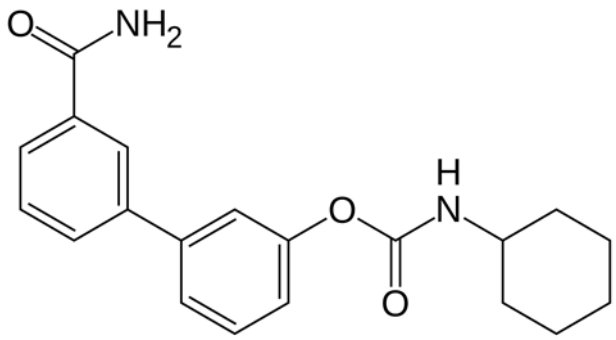


Figure 4.20. Chemical structure of URB597, also known as KDS-4103.

The FAAH inhibitor showed no significant effect against cephalosporinase from *E. cloacae* sp. (Fig. 4.21). The graph displays a slight decrease in the AmpC enzyme activity, especially at concentrations 0.5 mM and 1 mM of URB597. It is necessary to mention that the concentrations used for URB597 were in the milimolar range, e.g., x1000 more concentrated than those used to assess the LY2183240 regioisomers.

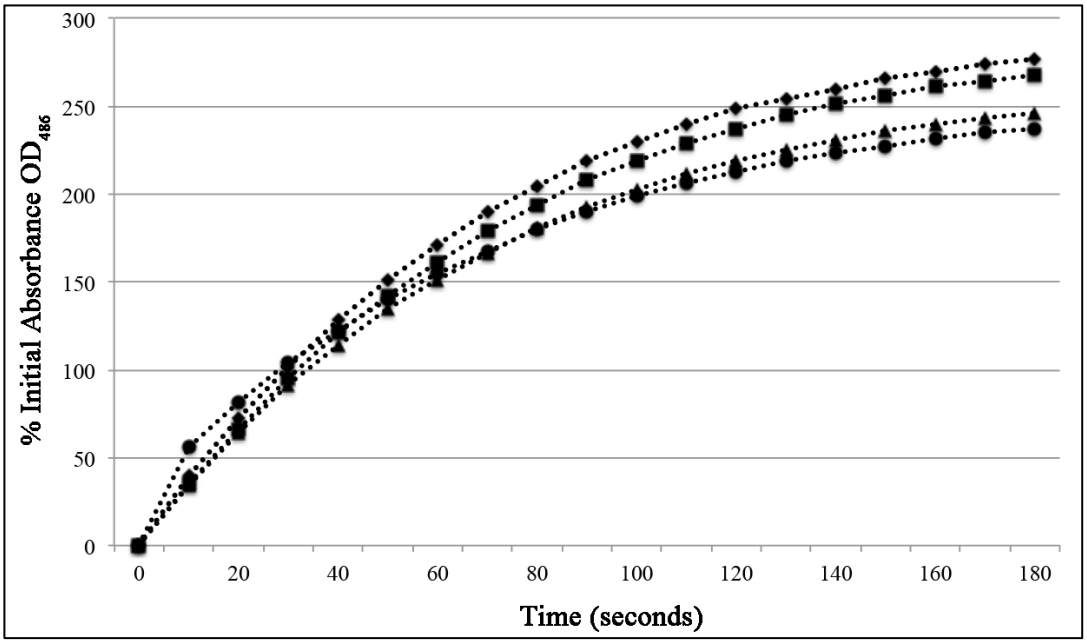


Figure 4.21. The effect of URB597 against a purified class C β-lactamase from *Enterobacter cloacae*. The mixture contained 2.5 nM of cephalosporinase with 0 mM (◆), 0.25 mM (■), 0.5 mM (▲) 1 mM (●) of URB597.

FAAH is an unusual serine hydrolase due to the catalysis of the hydrolysis of both amide and ester substrates at comparable rates, with an acylation rate limiting step (Mileni et al., 2010). Despite this, some classes of FAAH inhibitors also inhibit non-homologous serine hydrolases, suggesting the enzymes share some degree of mechanistic similarity. Nonetheless, in this study, the compound URB597 exhibited no relevant effect against class C β-lactamase from *E. cloacae*.

These findings suggest that although both URB597 and LY2183240 are FAAH inhibitors, only LY2183240 revealed activity against class C β-lactamase from *E. cloacae* sp. These data reinforce the idea that both LY2183240 regioisomers have promiscuous activities towards serine hydrolases, although to different extents.

4.3.4.5 IC_{50}s of Inhibitors

Half maximal inhibitory concentrations, IC_{50} values, for LY2183240 regioisomers were determined and compared with clavulanic acid, tazobactam and avibactam for both TEM-1 and AmpC β-lactamases (Table 15 and Figure 4.22). For both enzyme classes, avibactam presented the lowest IC_{50} values, 0.01 and 0.11 µM for class A and C, respectively. For the class A enzyme TEM-1, clavulanic acid gave an IC_{50} value of 0.37 µM. Tazobactam had IC_{50} values of 0.03 and 0.57 µM, for TEM-1 and

AmpC, respectively. Both regioisomers gave rise to similar IC_{50} values; the 1,5-isomer showed a slight higher activity than the 2,5-isomer towards TEM-1 with values of 244 and 290 μM, respectively. These high IC_{50} values exhibited by both regioisomers towards the class A β-lactamase corroborates with previous experiments on TEM-1 (Section 4.3.4.1).

Regarding the enzyme AmpC, tazobactam and avibactam, revealed higher IC_{50} values when compared to the class A TEM-1 β -lactamase. On the other hand, both LY2183240 regioisomers showed significantly lower IC_{50} values for this class of enzyme, exhibiting 20- to 30-fold higher activity when compared with the class A β-lactamase, illustrating a high specific activity for class C β-lactamases.

Table 15: Half maximal inhibitory concentration values (μM) for LY2183240 regioisomers and other β-lactamase inhibitors determined after 10 minutes of incubation with TEM-1 or AmpC.

			IC_{50} (μM)*				
Enzyme	Class	Source	Clav. acid	Tazobactam	Avibactam	1,5-	2,5-
TEM-1	A	*E. coli*	0.37	0.03	0.01	244	290
AmpC	C	*E. cloacae*	N.D.	0.57	0.11	6.78	13.83

* Performed in triplicate.
N.D. – Not determined.

An extensive search in the literature, including ChEMBL, one of the major databases of public bioactivities for small molecules, revealed substantial variations in the IC_{50} values from several different studies, employing the same reference compounds and similar β-lactamases used in this study (Bento et al., 2014).

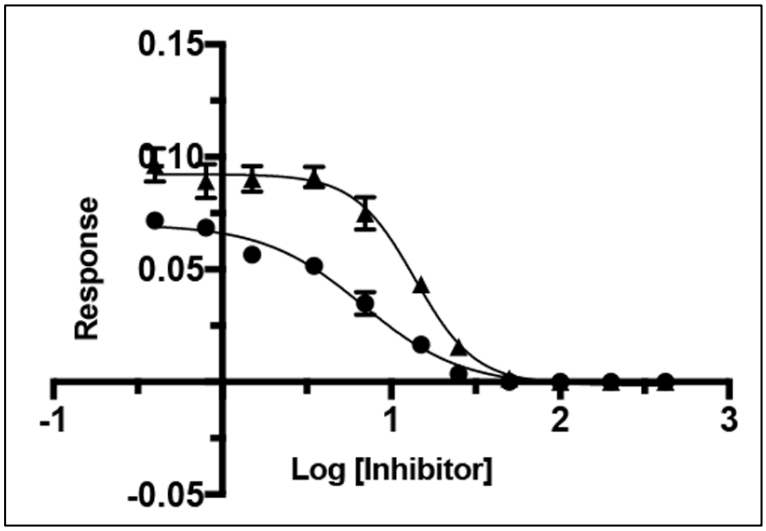


Figure 4.22. Determination of IC_{50} values for LY2183240 regioisomers against Ampc β-lactamase from *E. cloacae*; 1,5-isomer (●); 2,5-isomer (▲). The point of inflection of this curve corresponds to the logarithm of the inhibitor concentration that decreases enzyme velocity (*V*) by 50 %.

For instance, with the enzyme TEM-1, Payne et al. (1994) reported an IC_{50} for tazobactam of 0.04 µM, similar to that found in this study (0.03 µM). Conversely, wide-ranging IC_{50} values, from 0.01 to 0.1 µM, were also reported in the literature (Bush et al., 1993; Bret et al., 1997; Chaibi et al., 1998).

In a comparable way, some variations seem to occur with the inhibitor clavulanic acid as well. Many studies reported different IC_{50} values in a range of 0.02 to 0.12 µM (Payne et al., 1994; Bret et al., 1997; Caniça et al., 1998; Jamieson et al., 2003).

With regard to the AmpC enzyme, similar variations were reported, presenting divergences in the half maximal inhibitory concentrations to all standard compounds used. In the case of tazobactam, for example, there are reports showing IC_{50} values fluctuating from 0.0085 to 25 µM (Bush et al., 1993; Doi et al., 2004).

These variances can be attributed to several factors, including the method used and enzyme employed. However, one of the main aspects is the fact that IC_{50} data is very assay specific and comparable only under certain conditions (Kalliokoski et al., 2013).

In this context, in order to establish robust data regarding β-lactamase inhibitory activities, an extensive kinetic study using the same compounds and enzymes, was performed. Moreover, the relation between IC_{50} and K_i (Constant of Inhibition) was evaluated and further discussed in the following sections.

4.3.4.6 Inactivation of the AmpC β-lactamase from **E. cloacae** *by LY2183240 Regioisomers*

The main goal of this experiment was to verify the potential inhibitory activity of both LY2183240 isomers using the purified AmpC β-lactamase. For validation and comparison purposes, tazobactam and avibactam were used as reference standards.

Progressive and time-dependence inhibition of the class C β-lactamase (cephalosporinase) from *Enterobacter cloacae* by tazobactam is depicted in Figure 4.23. At the start, first-order of inhibition was observed up until 20 minutes of incubation, thereafter tazobactam inactivated almost completely the enzyme at two different inhibitor/enzyme (I/E) ratios, 33 and 165. Conversely, I/E ratios of 7 and 14, only led to a drop in enzyme activity of 60 % after approximately 2 hours of incubation. However, a further 18 hours of incubation resulted in a significant reduction in the enzyme activity to less than 20 %. There was no indication of a reversion of enzyme inhibition after 18 hours incubation for any of the concentrations tested.

These results are partially in accordance with a previous report using tazobactam and the class C β-lactamase from *Enterobacter cloacae* P99 (Bush et al., 1993). Bush and co-workers demonstrated a similar profile of enzyme inactivation by tazobactam, however, at an I/E ratio of 7:1 after 10 min of incubation 60 % of P99 enzyme activity was inhibited rather than 84 % found in this study. In addition, they revealed that the enzyme activity gradually recovered during incubation with different tazobactam concentrations, except at the highest concentration evaluated.

Throughout the 18 hours of incubation, the enzyme showed no significant loss in activity in the absence of the inhibitor in this study.

The lack of recovery from inhibition of the cephalosporinase after extended incubation (18h) observed in this study was not consistent with other studies that report partial recuperation of activity of *E. cloacae* β-lactamases with tazobactam (Akova et al., 1990; Bush et al., 1993). Notwithstanding, Akova et al. (1990) showed that the inactivated complexes were very stable in the recovery activity of the enzyme from *E. cloacae* inactivated by tazobactam. The authors suggested that the recovery kinetics fit with the accumulation of a single inactivated species that breaks down to free the active enzyme.

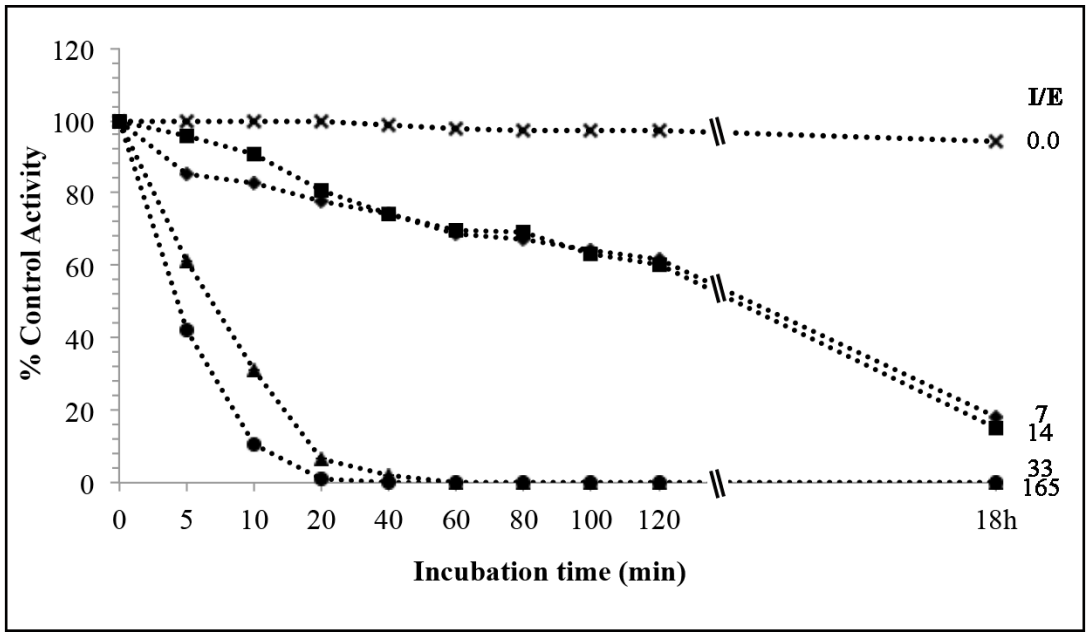


Figure 4.23. Inactivation of the class C β-lactamase from *Enterobacter cloacae* by tazobactam. The mixture contained β-lactamase (2.5 nM) with 18 (◆), 35 (■), 83 (▲), and 413 (●), nM tazobactam and a control (×) without the inhibitor present. I/E is the inhibitor-enzyme ratio.

In other words, the cephalosporinase can entirely be recovered only if the free inhibitor is removed; suggesting that the recovery occurred in these experiments only once the free tazobactam was separated by gel filtration. In this current work, inhibitor removal was not performed, representing a possible source of variance between the studies.

Although the recovery activity of the cephalosporinase used in this current work was entirely consistent, these minor differences observed may be related to the method employed or different enzymes studied.

In a similar fashion, avibactam demonstrated time- and concentration-dependent inhibitory activity of the cephalosporinase (Figure 4.24). After 5 minutes of incubation, the non-β-lactam inhibitor caused more than a 60% reduction in enzyme activity, and complete enzyme inactivation after 20 minutes of incubation at an inhibitor/ enzyme ratio of 60:1 (150 nM).

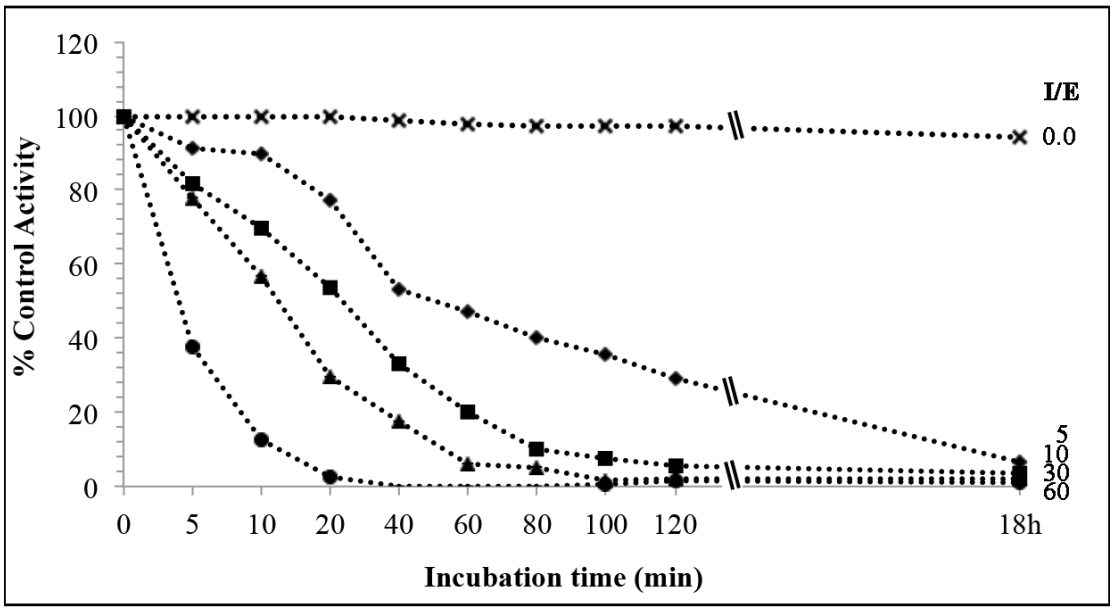


Figure 4.24. Inactivation of the class C β-lactamase from *Enterobacter cloacae* by avibactam. The mixture contained β-lactamase (2.5 nM) with 12 (◆), 25 (■), 75 (▲), and 150 (●), nM avibactam and a control without the inhibitor present (×). I/E is the inhibitor-enzyme ratio.

A gradual decrease in enzyme activity with 25 and 75 nM of avibactam was observed, with almost complete inactivation within 120 minutes of incubation.

It is worth mentioning that according to the literature, avibactam is a highly potent β-lactamase inhibitor. In this respect, lower concentrations and inhibitor/enzyme ratios were adopted in order to be able to compare the results with the others compounds evaluated.

Once again, no sign of enzyme reversibility was noted at all concentrations of avibactam tested. These results are somewhat similar to former reports; Stachyra et al. (2010) demonstrated a complete inactivation of the cephalosporinase P99 at a 1:1 I/E ratio after 5 and 30 minutes of incubation. Regarding enzyme recovery of activity, the same authors observed a recovery of less than 10 % activity after 10 to 15 minutes using 4-μM avibactam and the enzyme P99. However no further significant recovery of activity was observed within 24 hours.

The inhibitory activity of the LY2183240 regioisomers against AmpC β-lactamase was gradual showing time and concentration dependence. The rate of inhibition rose progressively as more inhibitor was provided (Figure 4.25). Isomer 1,5 showed complete inactivation of the enzyme after 60 minutes of incubation at a molar ratio of 165:1 (Figure 4.25A). Comparatively, the 2,5 isomer exhibited a similar profile,

but to a lesser extent, with almost 80% of enzyme inactivation within the same time interval (Figure 4.25B). Furthermore, both isomers revealed no signs of reversibility throughout the incubation period assessed.

Once again, the influence of the position of the carbamoyl on the tetrazole is evident, illustrating that it may play an important role in β-lactamase inhibitory activity as well (Alexander & Cravatt, 2006; Ortar et al., 2007; Ortar et al., 2008; Moore et al., 2005). Although the inhibitor-enzyme molar ratios of 7 and 14 showed no significant signs of inhibition within the first 120 minutes, after 18 hours of incubation both concentrations did result in more than 50% of reduction of enzyme activity.

Ortar et al. (2008) demonstrated that several of the carbamoyl tetrazoles synthesized, including LY2183240 regioisomers, strongly inhibited the hydrolysis of 2-arachidonoyglycerol by a serine hydrolase (monoacylglycerol lipase-like) present in the cytosol of a fibroblast-like cell lines (COS cells). Moreover, the authors agreed only in part with the conclusions drawn by Alexander and Cravatt (2006) about the promiscuity of LY2183240, since their findings showed that out of the 17 tetrazoles screened, four compounds exhibited potent activity for FAAH and selectively over all other targets tested in the study.

From a chemical point of view, there are other reports in the literature highlighting the significance of covalent carbamoylation of the enzyme's serine nucleophile in the inhibitory activity of these compounds within mammalian cells (Adibekian et al., 2012; Ebdrup et al., 2004).

With regard to prokaryotic systems, avibactam, stands out as a novel and potent inhibitor of both class A and C β-lactamases (Stachyra et al., 2009). As mentioned above, avibactam is considered a non-β-lactam, and its chemical structure is composed of a bicyclic structure containing a strained cyclic urea, which includes a carbamoyl group (carboxamide) (See Figure 1.8D) (Choi et al., 2016).

Recently, several studies have outlined the potential mode of action of this compound towards β-lactamases (Stachyra et al., 2009; Ehmann et al., 2012; Lahiri et al., 2013; Lahiri et al., 2014; King et al., 2015). One of the defining features of the mechanism proposed is the stable acyl enzyme formed by the carbamoyl link between the inhibitor and the enzyme active-site serine residue (Lahiri et al., 2014).

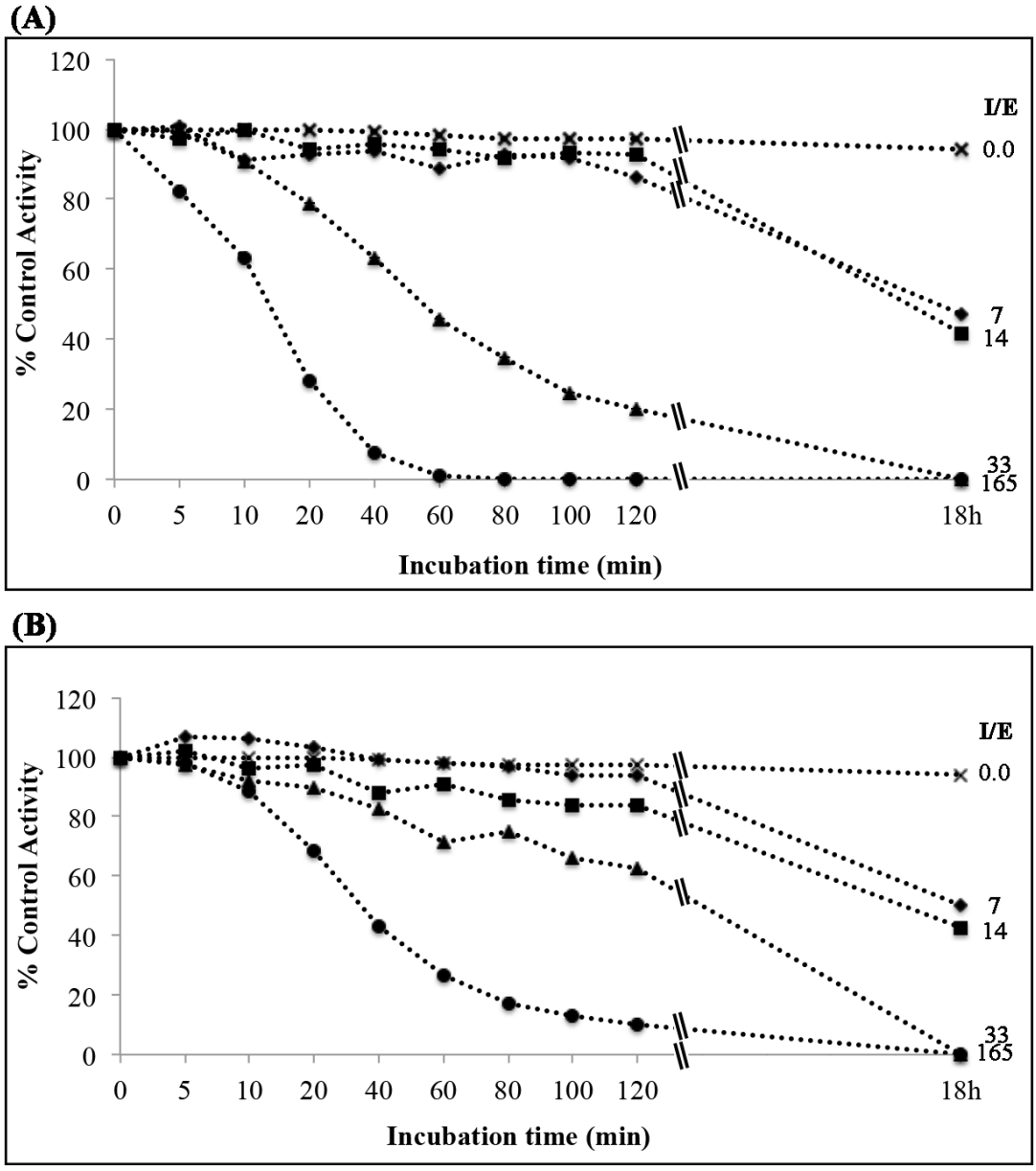


Figure 4.25. Inactivation of the class C β-lactamase from *Enterobacter cloacae* by LY2183240 regioisomers. **(A)** The mixture contained β-lactamase (2.5 nM) with 18 (◆), 35 (■), 83 (▲), and 413 (●), nM 1,5-LY2183240 and a negative control lacking the inhibitor (×). **(B)** The mixture contained β-lactamase (2.5 nM) with 18 (◆), 35 (■), 83 (▲), and 413 (●), nM 2,5-LY2183240 and a negative control in the absence of the inhibitor (×). I/E is the inhibitor-enzyme ratio.

Furthermore, according to Lahiri and co-workers (2014), the carboxamide of avibactam interacts with the side chains of Asn^{152} and Gln^{120} in the binding pocket of AmpC. Also, the inhibition of avibactam is believed to be reversible and the active inhibitor is regenerated via deacylation and recyclization of the 5-membered urea ring (Ehmann et al., 2012).

Clearly, the carbamoyl group can play an important role in the inhibitory activities of compounds towards serine hydrolases. Notwithstanding, further experimental investigations are needed to verify if the carbamoyl group present within LY2183240 regioisomers is involved in the inhibition of AmpC β-lactamases and this will be discussed in the following sections.

Although the study has successfully demonstrated the inhibitory effect of the compounds tested, it has certain limitations in terms of reversibility of the cephalosporinase. Unfortunately, it was not possible to detect any sign of enzyme reversibility with all the ligands assessed in this study. This may represent a critical restriction of this study, since this parameter helps in understanding the mode of action of the inhibitors. This factor will be investigated further and discussed in following sections.

An extensive research using several databases revealed that there are very few studies concerning LY2183240 in the literature. To the best of my knowledge, this is the first work reporting the activities of LY2183240 regioisomers against prokaryotic systems. The results presented in this section indicated that LY2183240 regioisomers possess strong inhibitory activities against class C β-lactamases. In the next section, the study will move on to discuss the potential mechanism of action of both LY2183240 regioisomers and explore the kinetic characteristics of this activity in more detail.

4.3.4.7 Kinetic Analysis of the Inhibition of Class C β-Lactamase from E. cloacae by LY2183240 Regioisomers

The purpose of a mechanism of action study is to characterize the interaction of a compound with its target to understand how natural substrates at physiologic concentrations will modulate this activity (Strelow et al., 2004).

Enzyme kinetic assays are widely used to estimate the substrate affinity and enzyme activity in general, including β-lactamases. The main purpose of this kinetic study is to verify the potency of LY2183240 regioisomers toward a purified class C β–lactamase and establish the modality of inhibition. In order to standardize and validate the method used in this project, three different standard β-lactamase inhibitors, clavulanic acid, tazobactam, and avibactam were used against two

different enzymes (TEM-1 and a purified AmpC from *Enterobacter cloacae*). In addition, in order to verify the range of LY2183240 regioisomer activities towards β-lactamases the metallo-β-lactamase NDM-1 was also tested.

To achieve this an appropriate experimental design for enzyme kinetic evaluation is essential for a correct interpretation of the data obtained. Estimating an inhibition constant (K_i) for a competitive inhibition model for instance, requires observations at low substrate concentrations, mainly because a competitive inhibitor is most effective at low substrate concentrations (Cornish-Bowden, 2014). From this standpoint, knowing from the literature the behavior of the standard β-lactamase inhibitors utilized in this work, low concentrations of the substrate nitrocefin (0 to 40 μM) were adopted. It is noteworthy that the enzyme concentrations employed were lower than the amount of substrate present in the solution, always having an excess of nitrocefin.

Figure 4.26 illustrates a plot of reaction velocity (V) as a function of substrate concentration [S] for the TEM-1 β-lactamase that follows Michaelis-Menten kinetics (Equation 3). In both reactions, the rate of catalysis was observed to gradually elevate as nitrocefin concentration increases. As expected, clavulanic acid gave rise to a significant reduction in the enzyme hydrolysis velocity in a concentration-dependent fashion (Figure 4.26A). In addition, tazobactam demonstrated a similar profile, although with a higher inhibitory potency, showing a reduction of a quarter of the TEM-1 hydrolytic activity, at the higher inhibitor concentration tested (Figure 4.26C).

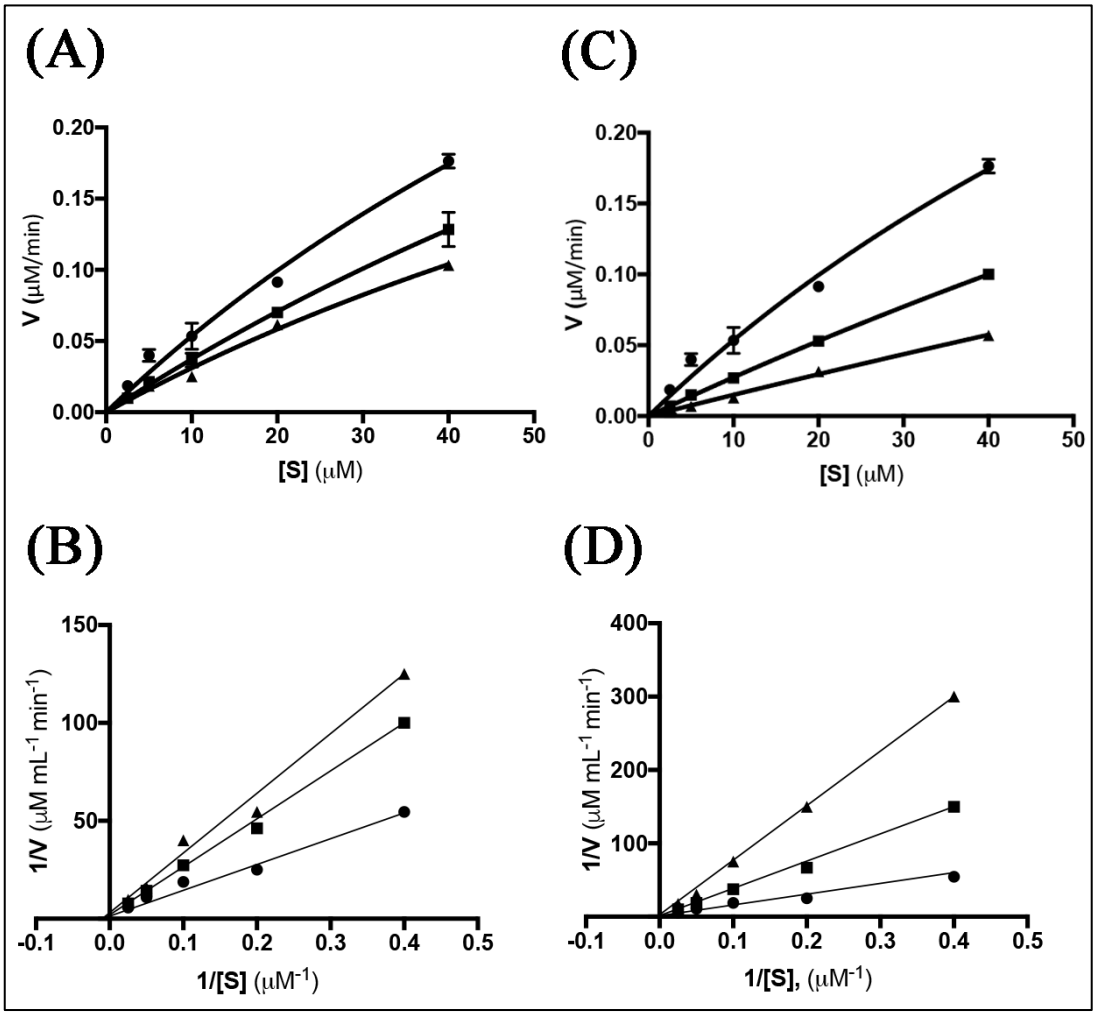


Figure 4.26. Determination of the inhibition mode of class A TEM-1 (0.25 μM) by clavulanic acid (A and B) and tazobactam (C and D). **(A)** Direct non-linear plot using the Michaelis-Menten equation. **(B)** Double reciprocal, Lineweaver-Burk plot. Final concentrations of clavulanic acid were 0 μM (●), 0.05 μM (■), 0.1 μM (▲). **(C)** Direct non-linear plot using the Michaelis-Menten equation. **(D)** Double reciprocal, Lineweaver-Burk plot. Final concentrations of tazobactam were 0 μM (●), 0.03 μM (■), 0.06 μM (▲).

To determine the mode of inhibition of TEM-1 by clavulanic acid and tazobactam, double reciprocal plots following the Lineweaver-Burk equation (Equation 4) in conjunction with the *alpha* (α) parameter derived from a mixed-model inhibition and statistical comparison by GraphPad Prism software were used (Minond et al., 2010). The results depicted in Figures 4.26 (B) and (D) suggest that both compounds are competitive inhibitors of β-lactamase class A TEM-1, e.g., the inhibitor acts as though it competes with the substrate, in this case nitrocefin, for binding to the enzyme (Copeland, 2000). Lineweaver-Burk plots are particularly valuable in

differentiating between competitive and noncompetitive inhibitors. In competitive inhibition, the intercept on the y-axis is the same in the presence or absence of inhibitor, although the slope is increased (Figure 4.26B & D) (Berg et al., 2002). The findings of the current study are consistent with former reports that studied clavulanate inhibition of class A β-lactamases (Labia & Peduzzi, 1978; Bret et al., 1997; Luhavaya & Grigorenko, 2010; Sulton et al., 2005; Drawz et al., 2010).

All the other relevant enzyme kinetic parameters for TEM-1 are summarized in Table 16. An increase in the Michaelis constant (apparent K_m) as a result of the presence of the inhibitors, clavulanic acid (170 ± 36.17) and tazobactam (299.3 ± 67.28), was observed (Table 16). In contrast, all the V_{max} values are similar, reinforcing again a competitive inhibition effect. This is in accordance with previously published determinations demonstrating the effect of a competitive inhibitor; a raised concentration of substrate is required to achieve a certain velocity, nonetheless the V_{max} is unaffected by a competitive inhibitor (Copeland, 2000). Another important parameter calculated in this study was the *alpha* (α) value. The constant α defines the level to which inhibitor binding influences the affinity of the enzyme for the substrate. The α-value for both standard inhibitors were greater than 1 (Table 16). According to Copeland (2000), this constant determines the mode of action, e.g., when α = 1 the inhibitor does not alter binding of substrate to the enzyme, being identical to noncompetitive inhibition. If α is very large (α > 1), binding of the inhibitor prevents the binding of the substrate, which is equivalent to competitive inhibition. When α is very small (but greater than zero), binding of the inhibitor enhances substrate binding to the enzyme, fitting nearly to the uncompetitive model. In the same way, statistical analyses of the comparison of fits ($p < 0.05$) indicate that the preferred model for both standard compounds is competitive inhibition.

Clavulanic acid and tazobactam revealed constants of inhibition (K_i) of 0.1113 ± 0.0145 µM and 0.0261 ± 0.0032 µM, respectively (Table 16). Similar results were reported for clavulanic acid towards TEM-1 with a K_i around 0.1 µM (Bret et al., 1997; Chaibi et al., 1998; Drawz et al., 2010; Imtiaz et al., 1994; Naumovski et al., 1992). Likewise, previous studies revealed a K_i between 0.01 to 0.02 µM for tazobactam (Bonomo et al., 1997; Chaibi et al., 1998; Drawz et al., 2010). The similarity of the constant of inhibition values from this study with former works reveals that the method employed is adequate and robust.

Table 16. Kinetic parameters of TEM-1 in presence of standard inhibitors.

	TEM-1		
	No inhibitor	**Clavulanic acid**	**Tazobactam**
K_m (μM)	122.5 ± 82.9	170 ± 36.17	299.3 ± 67.28
V_{max} (μM S^{-1})	0.7087 ± 0.3816	0.674 ± 0.1204	0.7167 ± 0.2319
K_{cat} (S^{-1})	2.83 ± 1.049	-	-
K_i (μM)	-	0.1113 ± 0.0145	0.0261 ± 0.0032
Alpha (α)	-	1.82 ± 4.89	18.63 ± 334.4
R^2	0.985	0.9903	0.9883
Preferred Model	-	Competitive ($p < 0.05$)	Competitive ($p < 0.05$)

A similar kinetic study with equivalent parameters was conducted utilizing the purified class C β-lactamase from *Enterobacter cloacae* with tazobactam and avibactam as reference compounds. In general, under the same conditions mentioned above, tazobactam showed an effective reduction in the enzyme hydrolysis velocity, however to a lesser extent when compared to the class A β-lactamase TEM-1 (Figure 4.27). This inhibitor revealed a similar comportment, consistent with a competitive inhibition model with K_i of 0.372 ± 0.064 μM (Table 17). Table 17 illustrates some of the main enzyme kinetic parameters for the cephalosporinase. Once again, as a competitive inhibitor, an increase in the K_m was observed. In comparison, tazobactam showed higher activity against TEM-1 than class C β-lactamase.

There are several lines of evidence that suggest that inhibitors of class A enzymes such as tazobactam have much less effect on AmpC β-lactamases (Jacoby, 2009). For that reason, few studies focus on tazobactam inhibition of class C β-lactamases. Nevertheless, data available reporting the constant of inhibition of tazobactam against AmpC β-lactamases is variable. In a report concerning the *in vitro* inhibition of CMY-type β-lactamases by tazobactam a K_i of 50 ± 9.5 μM was reported (Endimiani et al., 2010). In another study, Bush and co-workers (1993) demonstrated an IC_{50} of 8.5 nM towards a class C β-lactamase from *Enterobacter cloacae* P99.

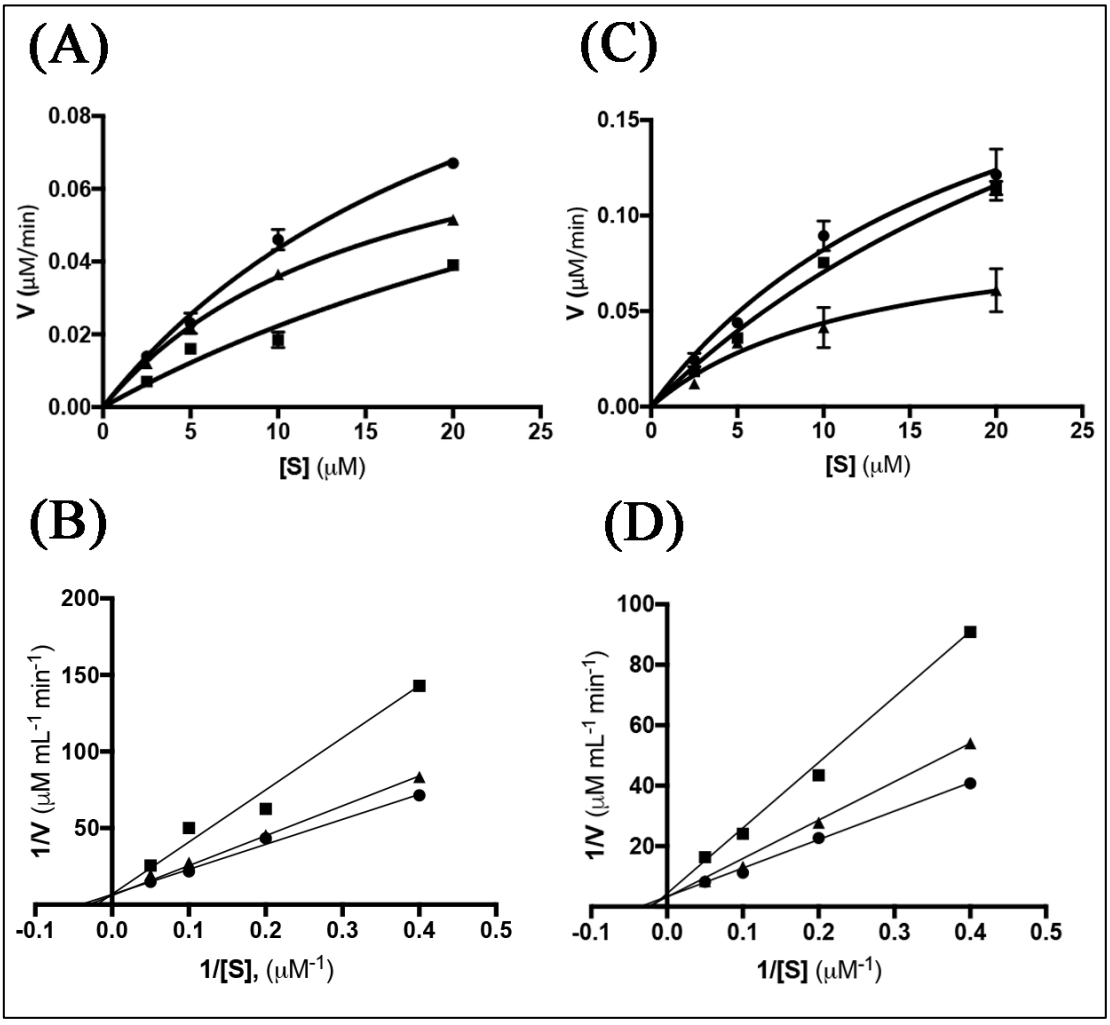


Figure 4.27. Competitive inhibition by standard β-lactamase inhibitors towards the class C enzyme from *Enterobacter cloacae*. **(A)** Direct non-linear plot of tazobactam using the Michaelis-Menten equation. **(B)** Double reciprocal, Lineweaver-Burk plot of tazobactam. Final concentrations of tazobactam were 0 μM (●), 0.2 μM (■), 0.5 μM (▲). **(C)** Direct non-linear plot of avibactam using the Michaelis-Menten equation. **(D)** Double reciprocal, Lineweaver-Burk plot of avibactam. Final concentrations of avibactam were 0 nM (●), 1 nM (▲), 5 nM (■).

Admittedly, although IC_{50} and K_i are not equivalent and challenging to compare, some of these differences still could be explained by certain factors. Firstly, although these enzymes belong to the same class, there are differences in physical and enzymatic properties, structures and essential active sites, among others that could affect directly inhibitor performance (Jacoby, 2009).

Table 17. Kinetic parameters of AmpC from *Enterobacter cloacae* in the presence of standard inhibitors and LY2183240 regioisomers.

	Cephalosporinase from *Enterobacter cloacae*				
	No inhibitor	**Tazobactam**	**Avibactam**	**1,5-LY2183240**	**2,5-LY2183240**
K_m (μM)	25.39 ± 1.506	49.09 ± 35.34	42.75 ± 16	31.1 ± 12.25	28.8 ± 0.9653
V_{max} (μM S^{-1})	0.1855 ± 0.008	0.129 ± 0.018	0.135 ± 0.018	0.227 ± 0.06	0.175 ± 0.004
K_{cat} (S^{-1})	742 ± 22.82	-	-	-	-
K_i (μM)	-	0.372 ± 0.064	0.0041 ± 0.002	1.8 ± 0.245	2.45 ± 0.301
Alpha (α)	-	1.054 ± 1.264	2.2 ± 4.9	6.42 ± 22.77	2.21 ± 1.9
R^2	0.9997	0.9888	0.9839	0.9897	0.9998
Preferred Model	-	Competitive [a]	Competitive [a]	Competitive [a]	Competitive [a]

[a] $p < 0.05$

Another important aspect is that the determination of K_i may not be uniform from laboratory to laboratory, including the method used. As a point of fact, there is an intriguing debate about how to characterize enzyme inhibition activity in terms of inhibition constants, such as IC_{50} and K_i (Cha, 1975). The aforementioned, 50 % inhibitory concentration (IC_{50}) measures the amount of inhibitor required to decrease enzyme activity to 50 % of its non-inhibited velocity and is often used in pharmacological studies to characterize inhibitors. While an IC_{50} can reflect an inhibitor's affinity, this parameter is not constantly consistent; for instance, an inhibitor can have a very poor affinity and acylate the enzyme slowly but still yield a low IC_{50} because of very low deacylation rates (Drawz et al., 2010). In addition, while K_i is a constant value for a given compound with an enzyme, an IC_{50} is a relative value, whose significance alters upon the concentration of substrate used in the assay. A particular compound will display several different IC_{50} values for the same enzyme if assays are performed employing different substrate concentrations (Burlingham & Widlanski, 2003). In this sense, the constant of inhibition K_i was selected as the main value in this study to estimate the potency of the compounds tested in all kinetic experiments to generate consistent and reliable data.

In a similar mode, albeit evidently more potent, avibactam exhibited a highly efficient decrease in the cephalosporinase hydrolysis velocity (Fig. 4.27C). Moreover, as predicted, the Lineweaver-Burk plot (Figure 4.27D) together with α value and statistical analysis revealed that avibactam acted as a competitive inhibitor with a low constant of inhibition of 4.1 ± 2 nM (Table 17).

The present findings regarding avibactam seem to be partially consistent with other research. In the last decade, an abundance of studies have been conducted with avibactam, formerly known as AVE1330A and NXL104 (Bonnefoy et al., 2004; Livermore et al., 2008; Stachyra et al., 2009; Mushtaq et al., 2010; Coleman, 2011; Ehmann et al., 2012; Ehmann et al., 2013; Lahiri et al., 2013; Lahiri et al., 2014; Endimiani et al., 2009). Several of these reports reveal the strong inhibitory activity of this non-β-lactam inhibitor towards relevant class A, C and D β-lactamases, especially when compared to other standard inhibitors such as clavulanic acid, tazobactam and sulbactam. Avibactam has lower IC_{50}s, in a range of 3 to 170 nM, and reduced reactivation rates, including cephalosporinases from *E. cloacae* and *P. aeruginosa* (Bonnefoy et al., 2004; Livermore et al., 2008; Stachyra et al., 2010; Lahiri et al., 2014). Furthermore, a mechanistic study of the inactivation of the class

C cephalosporinase P99 by avibactam revealed a constant of inhibition of 7.7 µM (Stachyra et al., 2010). This value is considerably higher compared with the value determined in this study (0.0041 µM). This discrepancy could be attributed to the methods used in both studies. Although similar, the K_i value was achieved by competition with nitrocefin during a very short incubation of 5 seconds. This brief incubation may have affected the potential activity of avibactam.

Avibactam and tazobactam were used in this study for the sole purpose of comparison and validation of the method, and the attainment of meticulous kinetic data for these standard compounds is out of the scope of this project. However the mode of action of avibactam will be discussed more in the following sections.

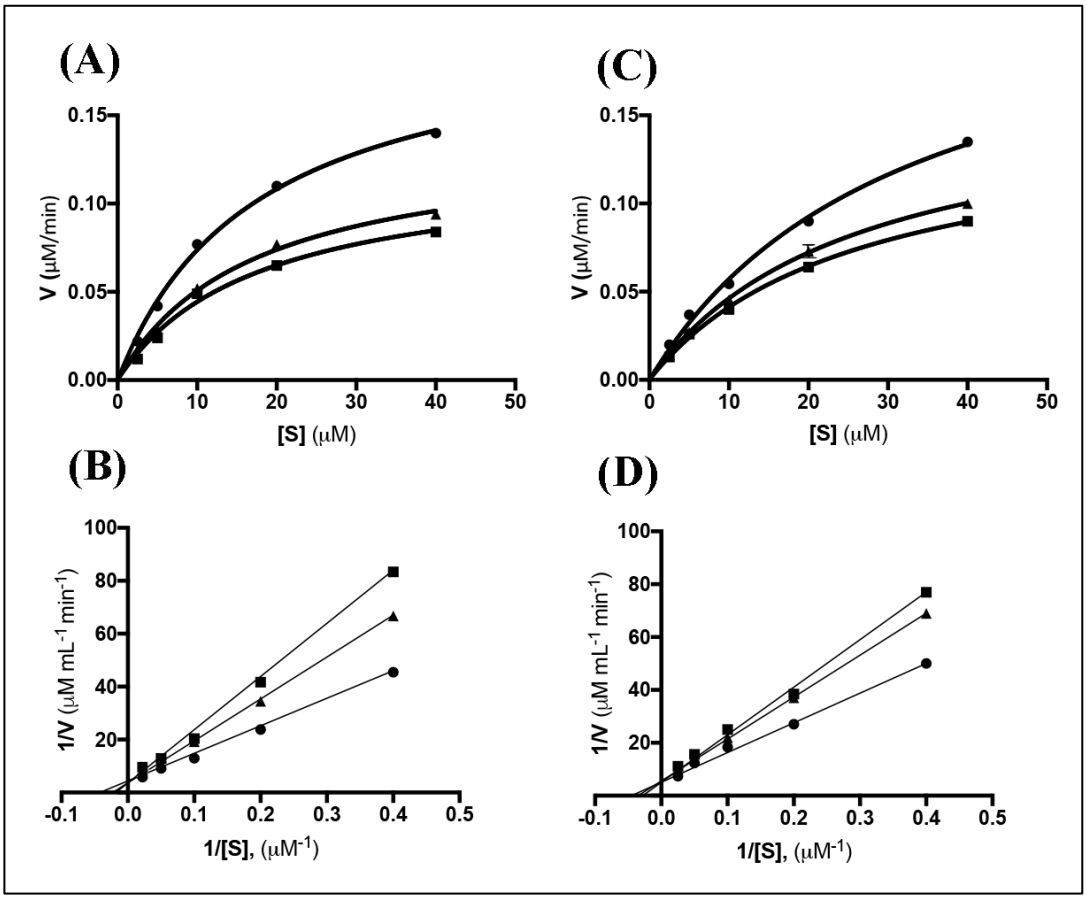


Figure 4.28. Competitive inhibition by LY2183240 regioisomers of the class C β-lactamase from *Enterobacter cloacae*. **(A)** 1,5-LY2183240 direct non-linear plot using the Michaelis-Menten equation. **(B)** 1,5-LY2183240 double reciprocal, Lineweaver-Burk plot. **(C)** 2,5-LY2183240 direct non-linear plot using the Michaelis-Menten equation. **(D)** 2,5-LY2183240 double reciprocal, Lineweaver-Burk plot. Final concentrations of both LY2183240 regioisomers were 0 µM (●), 0.5 µM (■), 0.8 µM (▲).

The kinetics analyses of both LY2183240 regioisomers using the Michaelis-Menten equation followed by the Lineweaver-Burk equation suggested that both isomers act as competitive inhibitors (Figure 4.28). Interestingly, both LY2183240 isomers gave rise to a significant reduction in the class C β-lactamase hydrolysis velocity. Also, an increase in K_m in the presence of the inhibitors, tazobactam (49.09 ± 35.34), avibactam (42.75 ± 16), 1,5-LY2183240 (31.1 ± 12.25) and 2,5-LY2183240 (28.8 ± 0.9653) was observed (Table 17) comparable to that observed for TEM-1 and cephalosporinase with the standard inhibitors. Nevertheless, contrary to expectations, a variation in some of the V_{max} values was detected. Both reference standards, tazobactam and avibactam, showed a significant decrease from 0.241 ± 0.0081 to 0.129 ± 0.0177 and 0.135 ± 0.018 in the V_{max} values, respectively. However, the 1,5-isomer had an increased V_{max}, whereas 2,5-LY exhibited a similar value when compared to control (no inhibitor). As mentioned above, in a competitive inhibition, the V_{max} value is unaffected. Despite the fact that the V_{max} values were not consistent, additional analysis of the inhibition model confirmed by the *alpha* parameter and the comparison of the fits ($p < 0.05$) suggested that the preferred model is competitive inhibition (Table 17).

Surprisingly, LY2183240 isomers demonstrated highly potent inhibitory activities against AmpC β-lactamase from *Enterobacter cloacae* with K_i values of 1.8 and 2.45 μM for 1,5- and 2,5-isomers, respectively. These values reveal these compounds to be less potent than tazobactam and avibactam assessed in this study, notwithstanding, LY2183240 was not developed to bind these types of enzymes, especially from a bacterial source. Furthermore, the primary modification of the LY2183240 regioisomers is the position of the carbonyl (1'5 to 2'5) on the tetrazole heterocyclic group (See Figure 1.17). Again, these data indicate that the change of position of this group increases the constant of inhibition K_i, suggesting an important role in the inhibitory effect towards the class C β-lactamase. As described previously, the 2,5-LY2183240 isomer displays a lower pharmacological potency as well as activity towards eukaryote serine hydrolases when compared to the 1,5-isomer (Moore et al., 2005; Alexander & Cravatt, 2006; Ortar et al., 2007; Ortar et al., 2008; Maione et al., 2009; Asada et al., 2015). Importantly, the mechanism of action of LY2183240 towards FAAH proposed by Alexander et al. (2006) involves the carbamylation of the enzyme's serine nucleophile (Ser^{241}), corroborating the significance of this group in inhibition of serine hydrolases.

Another parameter calculated in this kinetic study for both enzymes was K_{cat}. As mentioned above, this parameter is a constant that describes the turnover rate of an enzyme-substrate complex to product and enzyme. It is also the rate of catalysis with a particular substrate.

The class A β-lactamase TEM-1 exhibited a low K_{cat} value of 2.83 ± 1.049 s^{-1}. This finding is significantly different from previous results reported in the literature. The K_{cat} measurement for TEM-1 using nitrocefin as substrate reported by Legendre et al. (2002) was surprisingly 100 fold higher (300 s^{-1}), although determined under different experimental conditions and by different methods. One important factor that is not disclosed in Legendre's study is the enzyme concentration used, which would directly affect the constant K_{cat}. Another study conducted by Raquet and co-workers (1994) revealed an even higher K_{cat} value (930 s^{-1}) when compared to this current study. Possible explanations for the discrepancies in the catalytic properties of TEM-1 among these works mentioned are the different methodologies selected, as well as the enzyme source and concentrations. It is noteworthy that several studies do not indicate the amount of enzyme used in the assay, making it difficult to reproduce the method applied. Again, the concentration of enzyme reflects directly in the K_{cat} value (Equation 5), hence making it challenging to compare data of the same enzyme and inhibitors with previous studies.

On the other hand, AmpC β-lactamase from *Enterobacter cloacae* showed a high rate reaction of 742 ± 22.82 s^{-1} corroborating former studies performed on class C β-lactamases (Galleni et al., 1988; Siemers et al., 1996; Zindel et al., 2016). In a survey of the kinetic parameters of class C β-lactamases, two enzymes from *Enterobacter cloacae* (P99 and 908R) presented a K_{cat} of around 780 s^{-1} (Galleni et al., 1988). Moreover, in the same study, the enzymes showed a Michaelis constant (K_m) of around 25 μM, which support favorably the K_m value found in this current study. As expected, these data suggest a strong correlation between the constants K_m and K_{cat}.

Variations in K_{cat}, can be achieved by modifications in the enzyme, including modification of specific amino acid residues, or comparison of enzymes in different solution conditions like pH, ionic strength, and temperature, amongst others (Copeland, 2000; Segel, 1975). In addition, several enzyme kinetic studies commonly use the catalytic efficiency of an enzyme (K_{cat}/K_m) to compare the utilization of different substrates for a particular enzyme. This ratio has units of a second-order rate constant and is generally used to compare the efficiencies of

different enzymes to one another (Copeland, 2000; Segel, 1975; Cornish-Bowden, 2012). Intriguingly, there is a curious debate among enzyme kinetic researchers about the application and usefulness of the ratio K_{cat}/K_m. Eisenthal and co-workers (2007) showed that a particular enzyme even having a higher K_{cat}/K_m value (catalytic efficiency) is capable of catalyzing an equal reaction at lower rates than one possessing a lower catalytic efficiency, at specific substrate concentrations. This suggests that the ratio of the two reaction rates is not a constant, but depends on the value of $[S]/K_m$. Consequently, using catalytic efficiency value as an index for comparing the catalytic effectiveness of enzymes is not only incorrect, but can also be misleading (Eisenthal et al., 2007). However, the main purpose of this study is not the comparison of enzymatic efficiencies, but to verify the potency and modality of action of LY2183240 regioisomers towards β-lactamases. In this sense, it was decided not to use this parameter in this study because it was considered beyond the scope of this project.

To summarize, the kinetic study provided relevant and robust data in regards of the mode of action and potency of LY2183240 regioisomers towards class C β-lactamases. Although some divergences with other studies were noted, it was possible to confirm that both isomers act as competitive inhibitors and other important kinetic parameters were determined in a validated procedure. With the purpose of further studying this intriguing activity, an *in silico* study applying molecular docking was performed.

4.4 Conclusions

In this chapter, the 2,5-LY2183240 regioisomer has been shown to possess potent antimicrobial activity. This activity is selective towards certain Gram-positive bacteria, which includes *Staphylococcus* aureus (MSSA and MRSA) and *Bacillus subtilis*, but not *E. faecalis* or *S. pnuemoniae*. Conversely, the 1,5-LY2183240 isomer had no anti-bacterial activity strongly implicating the position of the carbamoyl on the tetrazole in the structure-activity relationship of the molecule. Studies with the membrane permeabilizing agent polyethyleneimine revealed that 2,5-LY2183240 had potential activity against *E. coli*, indicating that the selective activity of this compound may be related with the presence of the outer membrane in Gram-negative bacteria.

Further studies on the mechanism of antimicrobial activity suggest that while 2,5-LY2183240 had bacteriostatic activity this was probably not due to inhibition of protein synthesis, a target commonly associated with a cessation of growth rather than cell death. Despite specificity towards staphylococci, inhibition of teichoic acid synthesis (both cell wall and lipo-teichoic acids) was also unlikely to be responsible for the observed antimicrobial activity. Nevertheless, this activity may be related to the inhibition of bacterial fatty acid synthesis. Supporting this hypothesis, addition of exogenous fatty acids within Tween 80 was able to compromise 2-5-LY2183240 anti-staphylococcal activity. Due to redundancy, selective antimicrobial activity towards particular organisms is a feature of agents such as triclosan that target single enzymes involved in fatty acid synthesis. Based on the spectrum of activity and known redundancies at each of the steps in the fatty acid synthesis pathway, the most likely target was deduced to be FabI. However, characterization of a 2,5-LY2183240-resistant mutant revealed no alteration to the amino acid sequence of FabI or relevant changes to FabI protein expression, an observation confirmed by analysis of the *fabI* promoter region and western blot studies. Since resistance to 2,5-LY2183240 could be mediated through non-target related factors such as drug efflux or drug entry into the cell, FabI could not be ruled out as a potential target. In addition, whole cell protein profiling revealed the 2,5-LY2183240 mutant to have differences in the expressions of several proteins, suggesting resistance may occur through a more global effect. Due to the promiscuous nature of LY2183240, the

possibility of having multiple targets in *S. aureus* that collectively exhibit a bacteriostatic effect cannot be ruled out.

5 CHAPTER 5

In Silico and Crystallographic Studies of LY218340 Regioisomers and the AmpC β-Lactamase from *E. cloacae*

5.1 Introduction

The completion of the human genome project has resulted in an increasing number of new therapeutic targets for drug discovery. Furthermore, sequencing of bacterial genome sequences is now a standard procedure, and the information from tens of thousands of bacterial genomes has had a major impact on the view of the bacterial world, including potential novel antimicrobial targets (Land et al., 2015). At the same time, high-throughput protein purification, X-ray crystallography and mass spectroscopy techniques have been developed and contributed to many structural details of proteins and protein–ligand complexes (Walters et al., 1998; Langer & Hoffmann, 2001; Bajorath, 2002; Kitchen et al., 2004; Jorgensen, 2004). These advances allow the computational strategies to permeate all aspects of drug discovery today, such as the virtual screening techniques for hit identification and methods for lead optimization (Gohlke & Klebe, 2002; Meng et al., 2011). Compared with traditional experimental high-throughput screening *in silico* studies may be a more direct and rational drug discovery approach and has the advantage of low cost and effective screening (Moitessier et al., 2009). Moreover, molecular docking approach can be used to model the interaction between a small molecule and a protein at the atomic level, which allows characterisation of the behavior of small molecules in the binding site of target proteins as well as the elucidation of fundamental biochemical processes (McConkey et al., 2002).

This chapter will further investigate the mechanism of action of the inhibitory activity of LY2183240 regioisomers towards AmpC β-lactamases using molecular modeling, mass spectrometry and X-ray crystallography.

5.2 Objectives

The main objective of this chapter is to evaluate the interactions of the two LY2183240 regioisomers with the AmpC β-lactamase of *E. cloacae* using molecular modeling and crystallography.

The specific aims of the study are as follows:

- To evaluate the potential mechanism of action of the β-lactamase inhibitory activity of LY2183240 regioisomers via an *in silico* study.
- To assess the interactions of LY2183240 regioisomers with the AmpC β-lactamase are of a covalent or non-covalent type using mass spectrometry.
- To determine the potential binding interactions of LY2183240 regioisomers with β-lactamases by X-ray crystallography.

5.3 Results and Discussion

5.3.1 *In silico* Study

Molecular docking is now considered a conventional approach in virtual screening or lead optimization for drug screening and design. This technique permits the study of the main interactions between different molecules using several software packages accessible for this kind of study (Grinter & Zou, 2014). The comprehension of the molecular system plays an important role in determining what software to apply in the study. On the whole, software packages can be categorized based on the molecular system that they are normally employed to analyses and may implement either rigid or flexible docking. In the case of iGEMDOCK, flexible docking is engaged, which means that the conformation of part or entire molecules change during the best-fit investigation (Hsu et al., 2011). Typically, molecular docking concerns two connected steps: sampling of possible conformational states of the protein-ligand complex as well as calculation of the free energy of such complexes, or producing a score that correlates with biological activity or other function, also known as scoring (Novikov & Chilov, 2009). In sampling, there is a generation of a variety of different conformations and poses of a ligand, including orientations regarding to the binding surface of the protein target. At the same time, the scoring function delineates the potential energy surface and ranks the predicted poses according to a mathematical algorithm. It is worth mentioning that the scoring function is where most software packages differ and where the most research has progressed (Jain, 2006; Huang et al., 2010; Yuriev et al., 2015; Barata et al., 2016). Frequently, docking software packages are assessed according to the accuracy of the free energy calculations and the pose predictions between protein and ligand. In general, the scoring functions can be grouped into three basic types according to how they are derived: force field-based, empirical, and knowledge-based (Wang et al., 2003; Huang et al., 2010). To begin with, force field-based scoring methods attempt to essentially calculate the atomic interaction energies in the system, for instance, van der Waals, bond terms (length, angles and torsions), and electrostatics derived from force-field parameters. Secondly, empirical scoring functions access parameter coefficients by fitting to many crystal complexes with identified binding affinities (Huang et al., 2010; Barata et al., 2016). These functions process the free energy

between protein and ligand into a sum of the weighted free binding energies for each component such as electrostatics and hydrogen bond, that contribute to the interaction (Wang et al., 2002). Lastly, knowledge-based functions are derived from a statistical analysis of the interaction distances among different pairs of atom types in co-crystallized protein-ligand structures. Subsequently, statistical analyses are employed to originate pairwise potentials acquired directly from experimental data. This approach derives the energy potentials for the atomic interactions of all residue pairs as a function of the distance between the involved atoms and consequently estimates the energies of all conformations (Sippl, 1990).

To further investigate the binding of LY2183240 regioisomers to the AmpC β-lactamase from *Enterobacter cloacae*, molecular docking of LY2183240 isomers was performed using the cephalosporinase P99 (PDB ID: 1xx2) which is a standard representative of the class C β-lactamase from *E. cloacae*.

Figure 5.1 illustrates the structure of a class C cephalosporinase from *E. cloacae* P99 utilized in this study. The crystal structure was selected through an investigation of the Protein Data Bank website. According to the deposition authors the crystal structure was obtained via vapor diffusion method using PEG 8,000, sodium cacodylate ($C_2H_7AsO_2$), $MgCl_2$, NaN_3, pH 6.5 - 8.5 (Charlier et al., 1983; Lobkovsky et al., 1993; Lobkovsky et al., 1994). Moreover, the method employed was X-ray diffraction with a resolution of 1.88 Å.

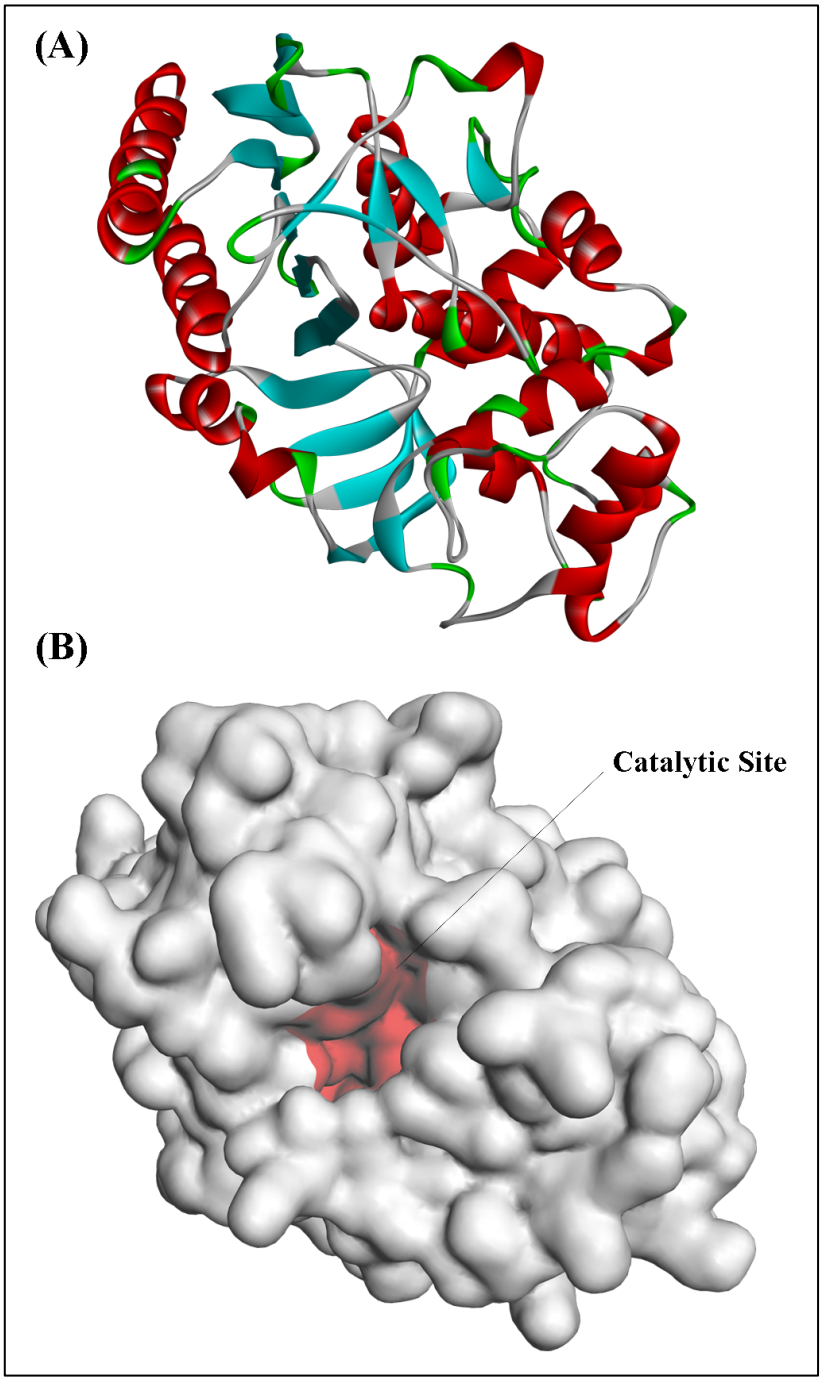


Figure 5.1. 3D Schematic representations of β-lactamase P99 structure (PBD 1xx2). **(A)** Ribbon diagram (in light blue for barrels and red for helices). This kind of visualization evidently shows the secondary and tertiary structure of the protein but completely hides atoms and in particular the side chain ones. **(B)** Molecular surface of the cephalosporinase showing the catalytic site in red.

The positioning of functional binding pockets on protein molecules is the basis of structural genomics and targeted drug discovery (Goldsmith-Fischman & Honig, 2003; Mirkovic et al., 2006; Weigelt et al., 2008; Van Voorhis et al., 2009; Hetényi & Van Der Spoel, 2011). Protein-ligand docking is not frequently applied to the whole protein surface to predict the binding site of a particular ligand. Therefore, most of the software demands some information about the binding site for accurate results. Albeit some packages permit for extension of the protein's area, typically by enlarging a grid or box, but this may result in loss of definition. For instance, with a larger area, reduced points inside the grid will be contemplated, consequently compromising the results (Barata et al., 2016). As an alternative, independent approaches or biological information about the protein target can be used to determine the principal binding site, and the following docking is confined to this specific site of interest. There are some linked methods in which the binding site search and docking procedure are performed concomitantly, also known as blind docking (Het Enyi & Van Der Spoel 2011; Grinter & Zou 2014). During this procedure the entire surface of the protein target is scanned for hypothetical binding pockets of the ligand, and an atomic resolution complex structure is developed (Hetényi & Van Der Spoel, 2011). Nevertheless, this method presents some limitations, for example, the requirement of using explicit water molecules to explore the binding site, as previously reported by Minke et al. (1999). Another disadvantage of blind docking is that the target molecule is not flexible, and the accuracy of the force field parameters is limited. In other words, the possibility of precisely docking a ligand to a protein target depends crucially on the quality of the target structure (Hetényi & van der Spoel, 2002).

In this sense, it is fundamental in this study to employ a software package without requiring information on the binding site, given that the mechanism of action of the LY2183240 regioisomers is still uncertain. Although the kinetic interactions studies with AmpC β-lactamase from *Enterobacter cloacae* and all the ligands indicate a competitive inhibition model, e.g., competing with the substrate for the same binding site, for this part of the work the iGEMDOCK software package was employed since the whole protein surface would be considered, hence the molecular docking would be entirely objective. Furthermore, molecular docking could have biased results if the ligand is restricted to interactions only within the active site.

Figure 5.2 illustrates all the poses predicted by the molecular docking for the five ligands used in this study. It was noticed that each ligand exhibited a range of different conformations and orientations in respect to the binding surface of β-lactamase P99. Overall, despite the fact that this method is targeting the entire protein surface, it was observed that most of the predicted conformations for all the ligands concentrated in the active site of P99.

As anticipated, nitrocefin, in yellow (Figure 5.2A), showed its poses restricted to the catalytic site. Similarly, avibactam, in orange (Figure 5.2B), exhibited all the conformations in the catalytic pocket. With regards to tazobactam (dark pink; Figure 5.2C), and both regioisomers (1,5-, cyan; 2,5-, green) (Figures 5.2D & E), it was noticed that iGEMDOCK identified hotspots mainly in the active site, but also on other parts of the protein surface. Although LY2183240 regioisomers exhibited poses outside of the catalytic site, the lowest energy conformations were interacting inside of the active site, as further discussed below.

Following onto the second stage of the docking procedure, these poses were scored and ranked according to a mathematical algorithm used to predict the binding affinities, in which the best candidates are those with the lowest energies or having individually high binding affinities. Due to some limitations in the ranking process of the package used, a re-scoring procedure was performed using VEGA ZZ software in order to circumvent the issue.

Table 18 depicts the energy rank of the all ligands used in the study. Additionally, the force-field scoring function of iGEMDOCK separated ligand binding energy into individual interaction terms such as van der Waals, hydrogen bond and electrostatic energy. The lowest energy solution, i.e., the maximum interaction, was found to be with the substrate nitrocefin. In addition, all the best poses with lowest energies of the five ligands were found to be in the active site of the protein, with similar residues interactions (Figures 5.3 to 5.7).

Among the inhibitors tested, the maximum interaction was observed for avibactam with a score of –114.34. By contrast, tazobactam showed the least interaction and, remarkably, both isomers of LY2183240 presented good interactions (Table 18).

The regioisomer 1,5-LY2183240 revealed a small lower energy solution of -109.09 kcal/mol when compared to the 2,5-isomer, -104,88 kcal/mol. Moreover, this difference can be related with the van der Walls interactions, since 1,5- and 2,5-isomers exhibited -95.08 and -81.83 kcal/mol, respectively. The present findings

seem to be consistent with the previous kinetic study data (Section 4.3.4.7) and inactivation curve (Section 4.3.4.6), showing that the potency of the 1,5-isomer is slightly higher towards AmpC β-lactamases.

Table 18. Docking scores for AmpC β-lactamase P99 (PDB 1xx2) rescored using VEGA ZZ.

Compound	Energy	van der Waals	HBond	Electrostatistic energy
Nitrocefin	-123.75	-84.33	-39.95	0.54
Avibactam	-114.34	-82.26	-32.08	0
1,5-LY2183240	-109.09	-95.08	-14.01	0
2,5-LY2183240	-104.88	-81.83	-23.05	0
Tazobactam	-93.17	-56.07	-35.97	-1.13

The units for all energy interactions are in kcal/mol.

Both LY2183240 regioisomers are larger compounds than tazobactam and consequently flexible molecules that bind more effectively within the enzyme active site resulting in higher binding energy. It is worth mentioning that the package iGEMDOCK provides algorithms for a flexible docking approach; which would show a larger decrease in total energy compared to the molecules in this study as these are smaller in structure and possess less flexibility. However, this seems not to be the case with the standard reference avibactam. The binding energies of the regioisomers are lower than tazobactam, but the kinetic analysis reveals that the compounds exhibited comparable inhibition (1,5-isomer K_i = 1.8 μM; 2,5-isomer K_i = 2.45 μM) to this reference inhibitor (K_i = 0.372 μM).

Fig 5.3 shows the interaction of the substrate nitrocefin and the P99 enzyme. As estimated, the predicted binding mode suggests that nitrocefin was more stable in the active site of this particular enzyme. The best pose calculated shows interaction between the oxygen of the β-lactam ring of nitrocefin with Ser^{64} (2.53 Å and 2.96 Å) via a carbon hydrogen bond. Furthermore, the other oxygen atoms of the carboxy group of nitrocefin strongly interact with the residues Tyr^{150} (2.31 Å), Thr^{316} (1.60

Å), Lys^{315} (2.11 Å) and His^{314} (1.71 Å) by conventional hydrogen bonds. Finally, it was also noticed that a π-cation interaction between the 2-thienylacetyl-amino ring and the residue Arg^{349} (3.92 Å), and an alkil/π-alkil interactions with residues Leu^{293} (4.56 Å) and Ala^{292} (4.22 Å) were predicted.

All the ligand-protein interactions distances of the ligands tested are summarized in Table 19.

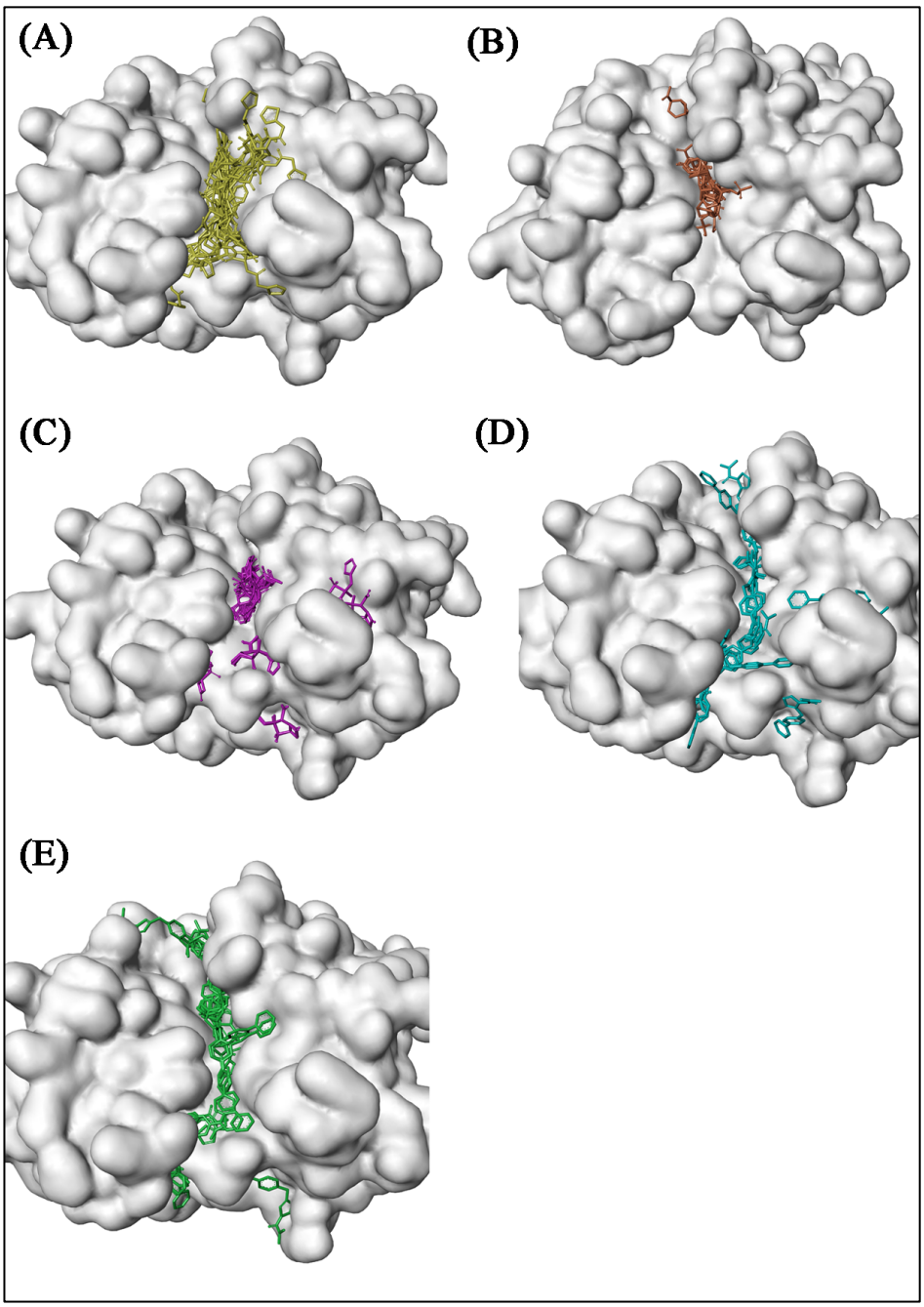


Figure 5.2. All binding modes predicted by molecular docking using iGEMDOCK package software targeting the whole β-lactamase P99 surface (20 solutions). **(A)** Nitrocefin (substrate), **(B)** Avibactam, **(C)** Tazobactam, **(D)** 1,5-LY2183240, **(E)** 2,5-LY2183240.

Figure 5.4 depicts one of the predicted conformations of avibactam interacting with the cephalosporinase P99. The carboxamide group interacted with the residues Ser^{64} (2.56 Å and 2.16 Å) and Gly^{317} (2.61 Å and 2.79 Å) by van der Waals forces, Ser^{318} (2.81 Å and 2.38 Å) by conventional hydrogen bond and Leu^{119} (5.43 Å) by alkil/π-alkil interactions (Fig. 5.4A).

The sulfate moiety was positioned by a salt bridge and a π-anion interaction with the residue Thr^{316} (2.83 Å and 2.30 Å), together with Tyr^{150} (2.55 Å), Lys^{315} (2.86 Å and 2.69 Å) and Asn^{346} (2.46 Å) by a conventional hydrogen bond. In addition, Lys^{315} (2.49 Å) also presented a strong π-anion interaction with the oxygen of the sulfate group. It is worth mentioning that the lowest energy conformation of avibactam predicted by iGEMDOCK was not the best-docked pose, indicating some limitations of the software used. Nevertheless, after re-scoring process using Vega ZZ software, the lowest-energy conformation became the most favorable pose.

Figure 5.5 illustrates the main interactions of tazobactam and the β-lactamase P99. It was observed the triazole group strongly interacts with the residues Ser^{64} (2.67 Å) by π-lone pair interaction and Lys^{67} (4.32 Å) by a π-cation bond. The oxygen of the β-lactam ring interacted with Lys^{315} (2.63 Å) by hydrogen bond, and the residues Asn^{346} (3.26 Å) and Thr^{316} (2.32 Å) interacted with the oxygen of the carboxylic acid by hydrogen bond and van der Waals forces, respectively. Moreover, the molecular docking calculations suggested a conventional hydrogen bond between Tyr^{150} (3.35 Å) and the oxygen of the 4-thia-1-azabicyclo group of tazobactam. Also, a carbon interaction with Ser^{318} (3.25 Å) was noted.

The best pose predicted for the 1,5-isomer of LY2183240 by iGEMDOCK software and main interactions with the cephalosporinase P99 is represented in Figure 5.6. The tetrazole group of 1,5-LY2183240 exhibited interactions with the residues Tyr^{150} (4.39 Å), Lys^{315} (3.10 Å and 3.74 Å) and Ala^{292} (4.81 Å), showing a π-stacking (T-shaped), π-cation and π-sigma interactions, respectively. Also, the carbamoyl group interacted with the residue Thr^{316} (3.74 Å) by van der Waals forces. Moreover, interactions between the benzenes present in 1,5-LY2183240 and the residues Gly^{286} (2.78 Å), Met^{265} (4.38 Å), and Leu^{274} (4.57 Å) were observed.

Table 19. Heteroatom-heteroatom distances A – Z (Å) between the amino acids of β-lactamase P99 and all the ligands for the best conformations calculated.

Residue	Nitrocefin	Avibactam	Tazobactam	1,5-	2,5-
Ala^{292}	3.95	3.41	–	4.06	5.25
Ala^{292}	4.21	3.11	–	4.81	–
Ala^{292} [O]	–	–	–	–	3.43
Ala^{292} [O]	–	–	–	–	3.68
Arg^{148} [$^{\eta}$H]	–	2.12	–	–	–
Arg^{148} [$^{\eta}$H]	–	2.70	–	–	–
Arg^{349} [NH_2]	3.91	4.07	–	–	–
Asn^{152} [$^{\delta}$O]	–	–	3.35	–	–
Asn^{346} [$^{\delta}$O]	–	–	3.26	–	–
Glu^{272} [$^{\varepsilon}$O]	–	–	–	–	3.59
Glu^{272} [$^{\varepsilon}$O]	–	–	–	–	3.27
Glu^{272} [$^{\varepsilon}$O]	–	2.76	–	–	–
Glu^{272} [$^{\varepsilon}$O]	–	–	–	3.84	–
Gly^{286} [C,O]; Ser^{287} [N]	–	–	–		3.35
Gly^{286} [C,O]; Ser^{287} [N]	–	–	–	3.39	–
Gly^{286} [$^{\alpha}$H]	–	–	–	2.77	–
His^{314}	–	–	–	–	5.31
His^{314} [$^{\varepsilon}$H]	1.71	–	–	–	–
Leu^{274}	–	–	–	4.56	5.47
Leu^{293}	4.56	–	–	–	–
Leu^{293}	–	4.28	–	–	–
Lys^{315}	–	–	–	–	5.28
Lys^{315} [$^{\alpha}$H]	2.11	–	–	–	–
Lys^{315} [$^{\zeta}$H]	2.35	2.42	–	–	2.07
Lys^{315} [$^{\zeta}$H]	1.58	–	–	–	–
Lys^{315} [$^{\zeta}$H]	2.85	–	2.62	3.09	–
Lys^{67} [$^{\zeta}$N]	–	–	4.32	–	–
Met^{265}	–	–	–	4.37	–
Met^{265}	–	–	–	–	5.27
Met^{265}	–	–	–	–	3.85
Ser^{318} [H]	–	2.64	–	–	–
Ser^{318} [O]	–	–	3.24	–	–
Ser^{318} [$^{\beta}$H]	–	2.85	–	–	–
Ser^{64} [$^{\beta}$H]	2.95	–	–	–	–
Ser^{64} [$^{\beta}$H]	2.52	–	–	–	–
Ser^{64} [$^{\beta}$H]	–	3.09	–	–	–
Ser^{64} [$^{\gamma}$O]	–	–	2.66	–	–
Thr^{316} [O]	–	–	–	3.73	–
Thr^{316} [H]	1.60	–	–	–	–

Table 19. (Continuation) Heteroatom-heteroatom distances A – Z (Å) between the amino acids of β-lactamase P99 and all the ligands for the best conformations calculated.

Residue	Nitrocefin	Avibactam	Tazobactam	1,5-	2,5-
Thr^{316} [H]	–	–	–	–	2.97
Thr^{316} [O]	3.37	–	–	–	–
Thr^{316} [$^{\beta}$H]	–	2.04	–	–	–
Thr^{316} [$^{\beta}$H]	–	–	2.31	–	–
Thr^{316} [$^{\gamma}$O]	–	–	–	–	2.92
Tyr^{150}	–	4.27	–	4.39	3.45
Tyr^{150} [$^{\eta}$H]	2.31	2.56	2.48	–	–

In Figure 5.7, the enzyme-substrate interactions between *E. cloacae* P99 protein and the 2,5-isomer of LY2183240 are indicated, as computed by molecular modelling. It was noticed that the 2,5-regioisomer revealed similar interactions when compared to 1,5-isomer. Essentially, the carbamoyl group interacted with Ala^{292} (3.43 Å and 3.68 Å) by carbon hydrogen bond, Lys^{315} (2.08 Å) by conventional hydrogen bond and Tyr^{150} (3.45 Å) by π-sigma interaction. In addition, the residues Glu^{272}, His^{314}, Thr^{316} and Lys^{315} interacted with the tetrazole group of 2,5-LY2183240 by π-anion, π-sigma, carbon hydrogen, and conventional hydrogen bonds, respectively. Still, both benzenes interacted by amide-π-stacked with the residue Gly^{286} and π-alkyl with the residues Met^{265} and Leu^{274}.

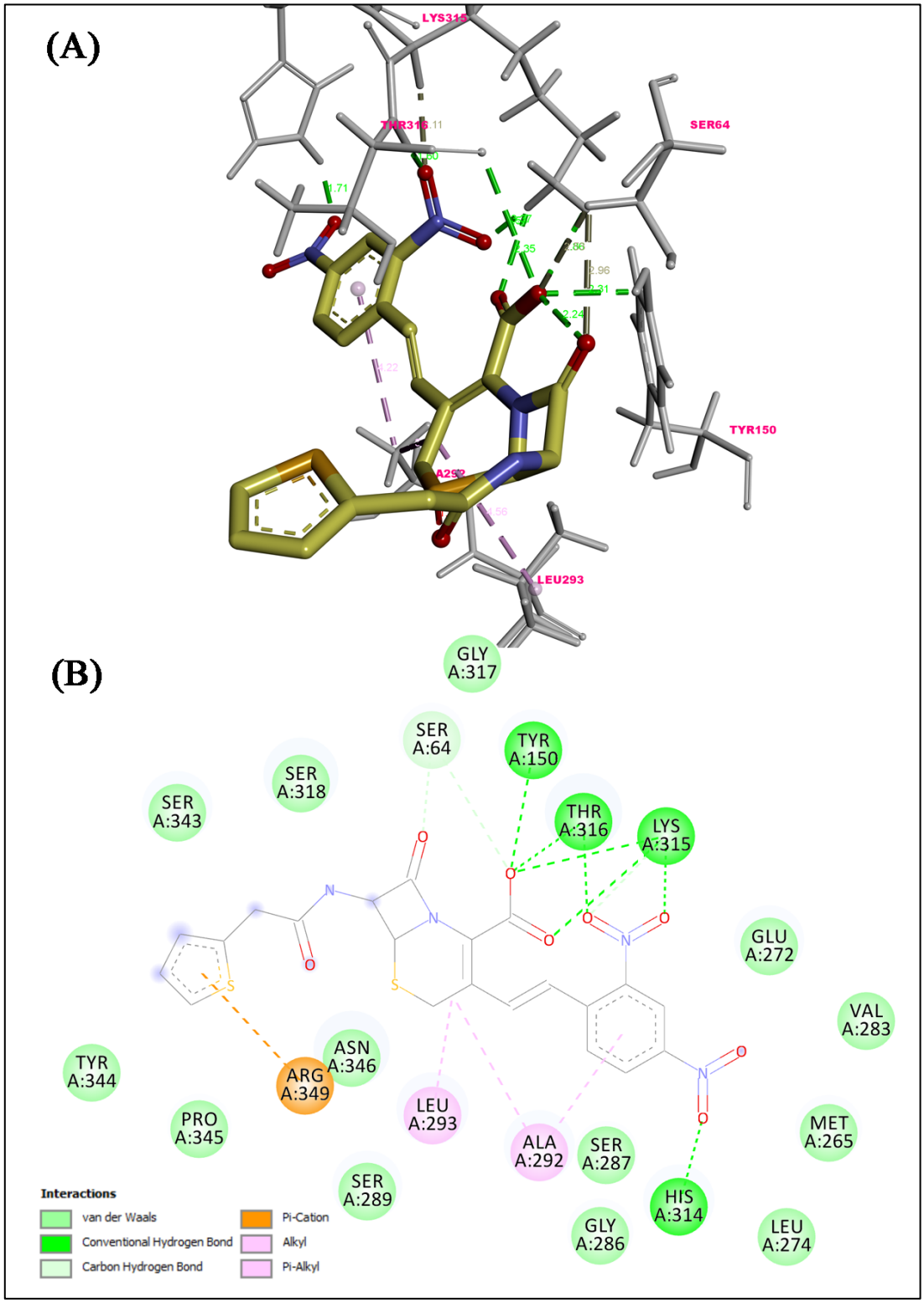


Figure 5.3. Nitrocefin interactions with β-lactamase P99. **(A)** Docking in the active site, nitrocefin appears in yellow; **(B)** 2D interactions.

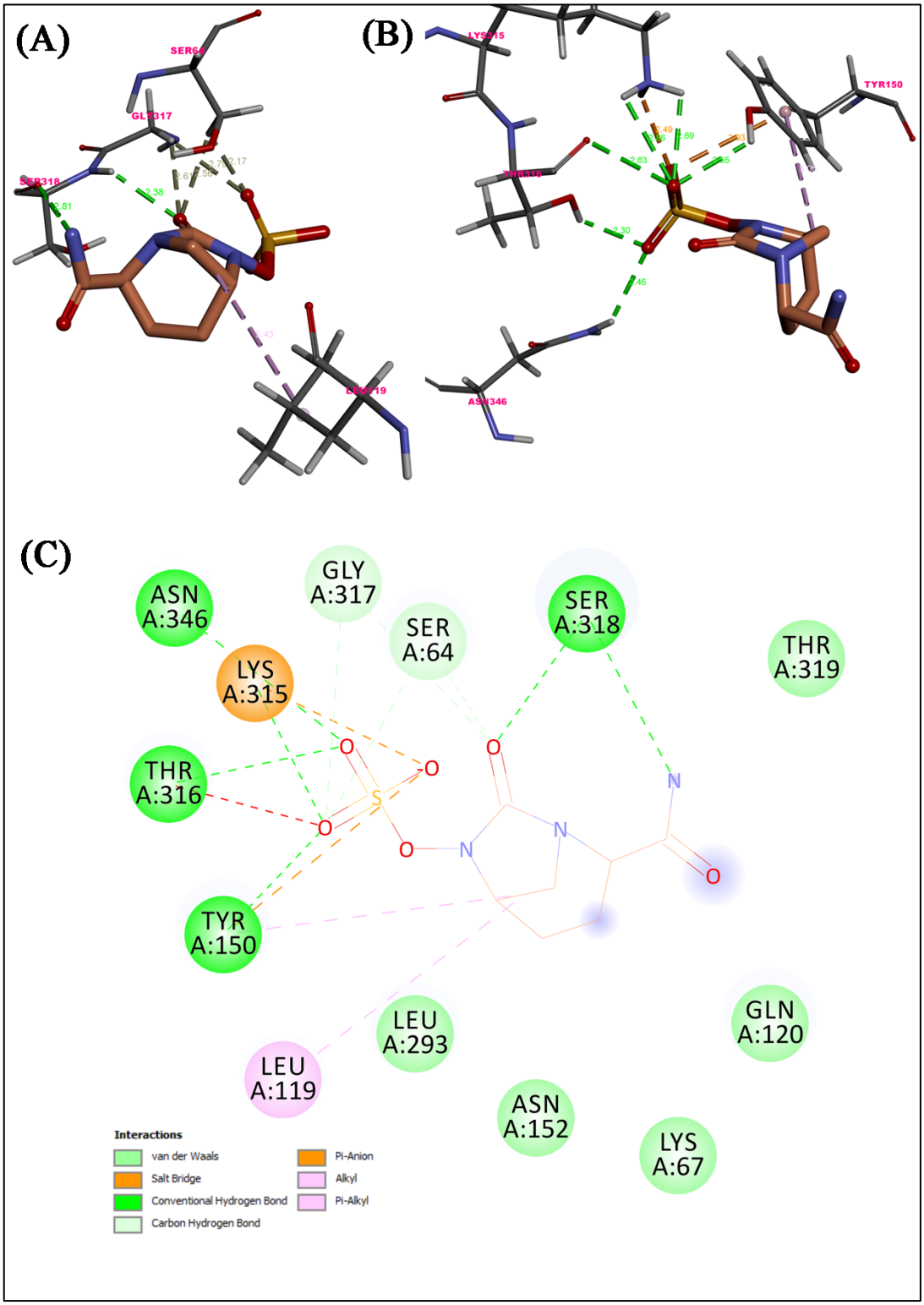


Figure 5.4. Avibactam interactions with β-lactamase P99. Different perspectives **(A)** and **(B)** of avibactam (orange) docking in the active site. **(C)** Interaction in 2D.

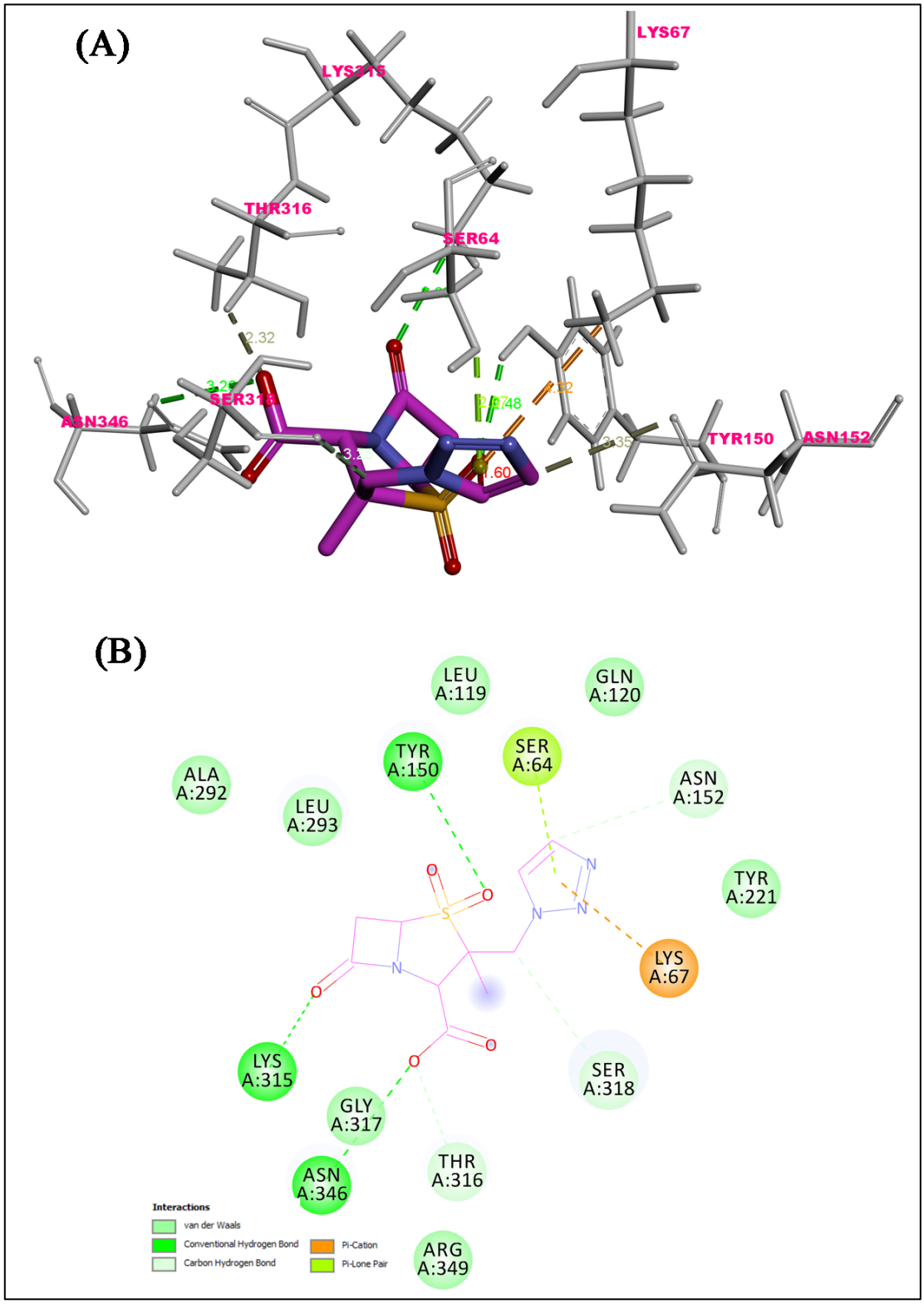


Figure 5.5. Tazobactam interactions with β-lactamase P99. **(A)** Docking in the active site, in magenta; **(B)** 2D interactions.

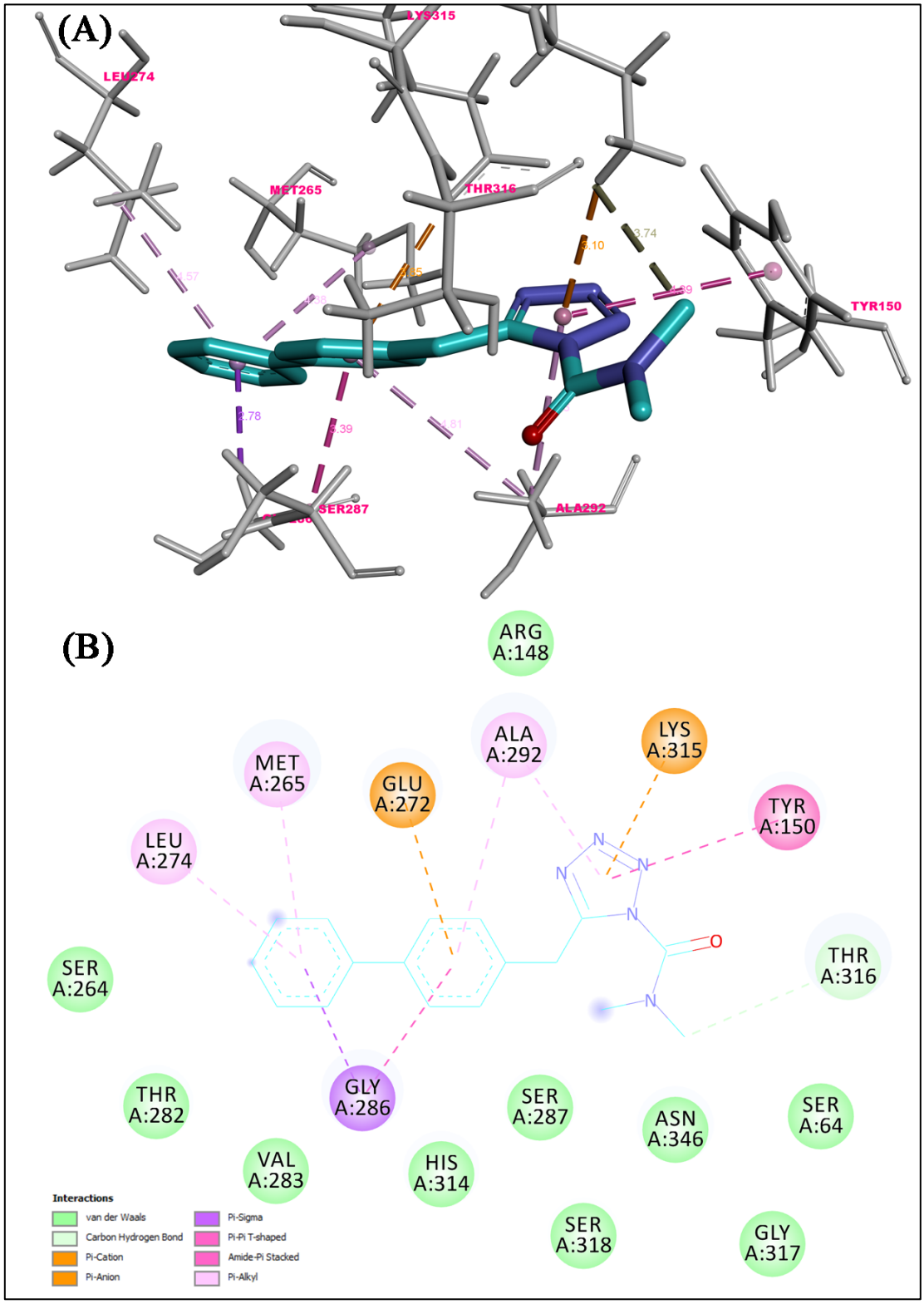


Figure 5.6. 1,5-LY2183240 interactions with β-lactamase P99. **(A)** Docking in the active site, in cyan; **(B)** 2D interactions.

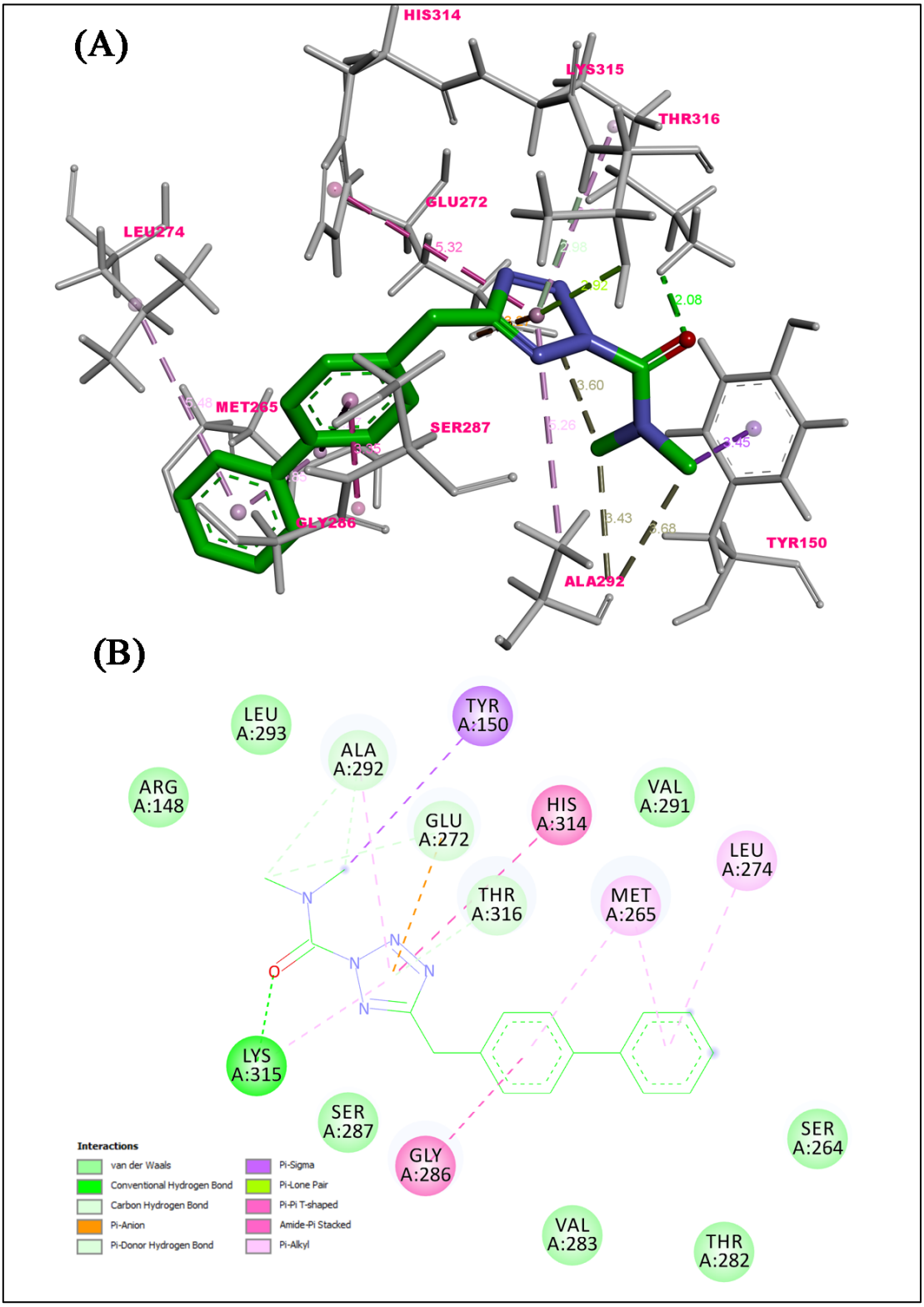


Figure 5.7. 2,5-LY2183240 interactions with β-lactamase P99. **(A)** Docking in the active site, in green; **(B)** 2D interactions.

For AmpC β-lactamases, several significant catalytic residues have been addressed. Many studies demonstrated that substitutions of catalytic amino acids such as Ser^{64}, Lys^{67}, Tyr^{150}, Asn^{152}, Thr^{316}, and Lys^{315} dramatically decrease enzyme activity up to 10^5-fold compared to the wild type (Tsukamoto et al., 1990; D Monnaie et al., 1994; Didier Monnaie et al., 1994; Dubus et al., 1994; Dubus et al., 1996; Lamotte-Brasseur et al., 2000; Beadle & Shoichet, 2002; Powers & Shoichet, 2002; Chen et al., 2006; Chen et al., 2009; Endimiani et al., 2010).

The current *in silico* study could demonstrate significant interactions with some of these major catalytic amino acids of AmpC β-lactamase P99.

Consistent with the literature, the substrate nitrocefin interacted with some of the major catalytic residues, including Ser^{64} (Figure 5.3). Moreover this substrate showed the lowest-energy pose among all the ligands tested. These findings substantiate former studies that described the characterisation of several key residues of cephalosporinases, like for example, the attack of residue Ser^{64} to the β-lactam ring (Oefner et al., 1990; Galleni et al., 1995; Knox et al., 1996; Curley & Pratt, 1997; Beadle & Shoichet, 2002; Chen et al., 2006). According with these studies, this reaction gradually advances initially by a pre-covalent first-encounter complex, secondly, two high-energy intermediates followed by a moderately low-energy covalent intermediate, and finally a product complex.

Other important residues that nitrocefin interacted with were Tyr^{150}, Lys^{315} and Thr^{316}. These residues present in the pocket sites of cephalosporinases play crucial roles as previously reported by many researchers (Oefner et al., 1990; D Monnaie et al., 1994; Dubus et al., 1994; Dubus et al., 1996; Tripathi & Nair, 2016; Beadle & Shoichet, 2002; Chen et al., 2006). Essentially, the residue Tyr^{150} seems to be the catalytic base for the deacylation process, receiving a proton from the deacylating water whilst it attacks the acyl-enzyme intermediate. More recent calculations have also proposed a conjugate-base hypothesis involving Tyr^{150} and Lys^{67}. Moreover, substitutions on Lys^{315} decreased activities considerably for both the acylation and deacylation steps, suggesting this residue is somehow involved in advancing the nucleophilic substitutions throughout this reaction pathway. In addition, the residue Thr^{316} along with Ser^{318} and Asn^{346} appears to perform an essential function in the substrate recognition, through their interactions with one of the oxygens of the β-lactam carboxyl group (Fenollar-Ferrer et al., 2003).

Disappointingly, no evidence of interactions with the catalytic residues Lys^{67} and Asn^{152} was observed. As a cephalosporin, nitrocefin possess a β-lactam ring required for the penicillin binding protein (PBP) reactivity, a 2-carboxyl group, an X-substituent and 3 and 7-substituents (See Figure 2.3). In a study conducted with cephalothin, a first-generation cephalosporin, and a class C β-lactamase, a general mechanism for the formation of an acyl−enzyme complex was proposed (Tripathi & Nair, 2013). The authors revealed the individual functions of the active site residues and the substrate, suggesting the important role of the Lys^{67} in the acylation step, while Tyr^{150} assists the protonation of the β-lactam nitrogen via either the substrate carboxylate group or a water molecule.

Other reports have shown that mutation of the conserved Asn^{152} can have a substantial effect on substrate selectivity of the enzyme AmpC (Lefurgy et al., 2007; Ruble et al., 2012; Skalweit et al., 2013).

As previously described throughout this thesis, recently, many works proposed the mechanism of action of avibactam at a molecular level, using different approaches, including crystallographic studies (Lahiri et al., 2014; King et al., 2015; Choi et al., 2016).

Particular to class C β-lactamases, these authors indicated that avibactam binds covalently with Ser^{64} in the active site of the enzyme. This molecule possesses a curious mode of action, acting as a covalent inhibitor via ring opening, however in contrast to other currently used β-lactamase inhibitors, this effect is reversible.

Lahiri and coworkers (2014) revealed that at least eight residues presented significant contributions to the binding interactions with avibactam. Particularly, the carboxamide moiety interacted with Asn^{152} and Gln^{120}, and the residues Thr^{316}, Lys^{315}, and Asn^{346} positioned the sulfate group while Tyr^{150} and Lys^{67} were placed to participate in catalytic functions to permit development of the covalent bond with Ser^{64}.

In comparison, the molecular docking data of avibactam provided in this current study is partially in agreement with these former reports. For instance, the sulfate group exhibited similar interactions with the same residues (Figure 5.4, Table 18). Conversely, in this particular case, crystallization and molecular docking are challenging to compare especially due to the fact that avibactam changed its structure and conformation after binding covalently to the residue Ser^{64}. Still, the best pose

predicted for avibactam by docking showed an approximate conformation in the active site reported by crystallization (Lahiri et al., 2014; King et al., 2015).

Not surprisingly, tazobactam, having a β-lactam ring, showed interactions with the main catalytic residues of the cephalosporinase P99. However, the interactions are not totally in agreement with the literature. The residue Ser^{64} showed a strong interaction with the triazole group of tazobactam. As mentioned above, many reports demonstrated that Ser^{64} directly attacks the β-lactam ring of the ligand (Chen et al., 2006). In this regard, the best-predicted pose of tazobactam seems to be inaccurate. Conversely, the interactions with the residues Tyr^{150}, Thr^{316} and Lys^{315} appear to be in accordance with previous reports (Fenollar-Ferrer et al., 2002; Fenollar-Ferrer et al., 2005; Chen et al., 2006)

Whilst some research has been carried out on LY2183240, no *in silico* study has investigated the activity of LY2183240 regioisomers against β-lactamases. The little research done with these compounds and other serine hydrolases as well as structurally similar molecules towards cephalosporinases may assist interpretation of the results obtained.

Contrary to expectations, the best poses of both LY2183240 regioisomers showed no significant interactions with Ser^{64} (Figures 5.6 and 5.7). None of the 20 solutions generated showed any interaction with this residue. These regioisomers are recognized for having promiscuous activities against several serine hydrolases and particularly to FAAH, exhibit a covalent bond with Ser^{241} (Alexander & Cravatt, 2006; Ortar et al., 2007). Therefore, the interaction with the amino acid Ser^{64} in the β-lactamase would be expected.

In comparison, the molecular docking performed with both LY2183240 regioisomers showed no significant differences between them, in the interactions toward β-lactamase P99. Both regioisomers presented comparable interactions with similar residues present in the active site of the enzyme (Table 18). However, it was observed that only the 2,5-isomer showed an interaction with the residue His^{314}. Notwithstanding, as previously demonstrated by Dubus and coworkers (1996) the His^{314} residue is insignificant and not required for catalysis.

Furthermore, previous reports suggest that the residues Tyr^{150}, Glu^{272} and Lys^{315} play a substantial role in class C β-lactamases (Dubus et al., 1996; D Monnaie et al., 1994). According to Dubus et al. (1996), residue Glu^{272} showed no essential function in the enzyme acylation process, however the amino acid was involved, to a minor

extent, in the deacylation. Notably, as cited before, the residue Tyr^{150} is suggested to be essential for catalysis, at least using different β-lactams as substrates. The authors proposed that the phenolate anion of Tyr^{150} together with the alkyl ammonium of Lys^{315}, acts as the general base responsible for the activation of the active site Ser^{64} during the acylation stage and, following activation of a water molecule, in the deacylation activity. Additionally, critical deficiency in hydrolytic capacity was revealed after the development of AmpC mutants, in which the residues Tyr^{150} and Lys^{315} were replaced by cysteine, suggesting the considerable contribution of these two residues in the catalytic reaction (Tsang & Leung, 2007).

In order to comprehend better the influence of the ligands in the catalytic site, Figure 5.8 shows the best binding modes predicted of LY2183240 regioisomers together with the substrate nitrocefin. Clearly, both isomers shared interactions with the same residues that interacted with the substrate nitrocefin, including Tyr^{150}, Thr^{316}, and Lys^{315}. This could indicate that these interactions may have a significant role in the competitive inhibitory effect of the regioisomers towards Class C β-lactamases. Moreover, it was observed that upon overlaying the conformations, the carbamoyl group of 1,5-LY2183240 was occupying the same space as the carboxy group of nitrocefin, interacting especially with Thr^{316}. This factor may be responsible for the higher inhibitory activity of 1,5-LY2183240 when compared to the 2,5-isomer (Figure 5.8B). Nevertheless, this hypothesis needs to be further confirmed with more studies.

Inevitably, there were some inaccuracies possibly due to the molecular docking and scoring function selected. The software iGEMDOCK, as with any other software package, has disadvantages and limitations that reflect in the final outcome. There are recurrent complications throughout *in silico* studies such as an inaccuracy in the binding site of the target, the docking pose selection, binding affinity, lack of clarity over whether the compound is an inhibitor or agonist, as well as the docking results being inconsistent with bioassays (Chen, 2015).

Genuinely, one of the main sources of error in the molecular docking applied in this study is the deletion of the water molecules prior to docking. As already mentioned, water molecules can promote many problems, for example, the process of finding poses and orientations or calculating binding affinity and due to these reasons it was decided to exclude them before the docking procedure. Nevertheless, as expected, water molecules can play important roles in the catalytic site of cephalosporinases. It

has been described that there are at least four water molecules in the active-binding site of AmpC enzymes (Yamaguchi et al., 2009). Powers and Shoichet (2002) suggested that one of the water molecules seems to be the deacylating water, which hydrogen bonds to the residue Thr^{316} along with a different water molecule. Furthermore, there is another water molecule that seems to have a hydrogen bond to the main-chain carbonyl of Ala^{318}. Finally, the last water molecule interacts with Ser^{64} and Ala^{318} (Yamaguchi et al., 2009).

Another limitation found in this study was the energy score ranking. The software iGEMDOCK gave an inaccurate ranking of the ligands (data not shown). Nevertheless, after rescoring a more precise energy ranking was presented. The reasons for this particular inaccuracy are still unknown, but other studies have reported similar divergences. For instance, free energy rescoring was more accurate compared with the selected package AutoDock4, indicating that free energy rescoring could be a beneficial tactic to reduce false positive calculations in molecular docking experiments (Malmstrom & Watowich, 2011). Moreover, the level at which the empirical energy function describes the observed free energy of binding impacts the accuracy of the relative energy difference between bound potential ligands, and consequently the correct identification of molecules that bind to the protein target.

An additional problem encountered in this molecular docking study was that the calculated avibactam best pose, i.e., showing the lowest energy (Table 18), did not corresponded with the best orientation previously found in the literature. However, among the 20 solutions predicted, one of them showed similar orientation with previous reports (Ehmann et al., 2013; Lahiri et al., 2014). Although avibactam is a covalent and reversible inhibitor as well described by several studies, in this current *in silico* study the software used only considered non-covalent bonding, neglecting potential covalent binding and therefore hindering the correlation with other reports.

The final limitation of this docking study is that it is restricted to non-covalent molecular docking. Recently, covalent molecular docking has been used successively by many researchers (London et al., 2014; London et al., 2015; Bianco et al., 2016). Notwithstanding, the larger part of the development research on docking methods is focused on the efficient prediction of the binding modes of non-covalent inhibitors (Kumalo et al., 2015). The ligands that bind covalently to the receptor are complex and problematic; a key problem of covalent inhibitors is their off-target reactivity

because of the presence of electrophilic reactive groups. In addition, covalent docking possesses similar problems to noncovalent docking, including poor scoring functions, with entropy, speed and accuracy (Kumalo et al., 2015). In this respect, the decision of what type of docking program is the best for a specific target protein is challenging and intricate (Chen, 2015).

The primary goal of this *in silico* study was to verify the potential mechanism of action of both LY2183240 regioisomers towards cephalosporinases as well as confirm the inhibitory activity model found in the kinetic assay. Therefore, a deep and extensive analysis of the molecular docking study is beyond of the scope of this thesis.

In this context, apart from the limitations presented and some discordances with literature, these findings do support the earlier findings in this study. Molecular modelling studies corroborate the hypothesis that LY2183240 regioisomers bind within the active site of class C β-lactamases, interacting with some of the same residues used by the substrate nitrocefin and standard β-lactamase inhibitors. In addition, these results support the competitive inhibition model previously observed in the kinetic study.

With this in mind, assessment of a potential covalent complex of LY2183240 regioisomers and AmpC β-lactamase is essential to confirm the proposed binding mode. Hence, in the next section assessment of the potential production of a covalent enzyme-inhibitor complex was determined.

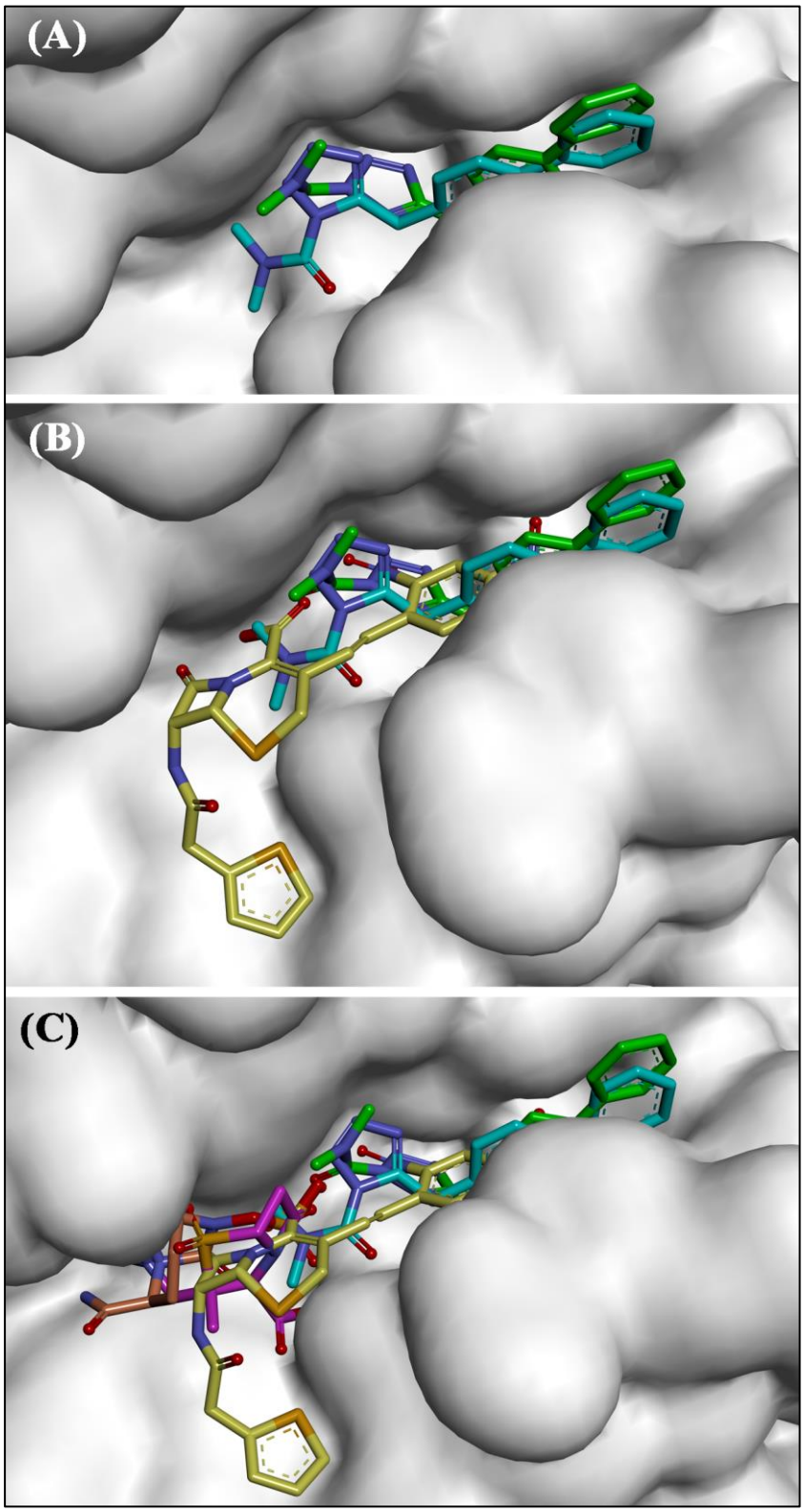


Figure 5.8. Best poses of molecular docking of **(A)** LY2183240 regioisomers 1,5 (cyan) and 2,5 (green), **(B)** LY2183240 regioisomers 1,5 (cyan) and 2,5 (green) with nitrocefin (yellow), and **(C)** all ligands used in this study; LY2183240 regioisomers 1,5 (cyan) and 2,5 (green) with nitrocefin (yellow), avibactam (orange) and tazobactam (dark pink).

5.3.2 LY2183240 Regioisomers Revealed a Non-Covalent Interaction with AmpC β-Lactamase

The use of mass spectrometry in proteomics is divided into three main fields. It is the preferred method for characterization and quality control of recombinant proteins and other macromolecules, a relevant assignment in the area of biotechnology. Mass spectrometry is also frequently used for protein identification, including large-scale proteomic projects. Lastly, due to the fact that mass spectrometry measures the molecular weight of a protein, it is the technique of choice for the detection and characterization of posttranslational modifications and can identify any covalent modification that alters the mass of a protein (Mann et al., 2001; Yanes et al., 2004).
In this respect, mass spectrometry is an appealing substitute to other procedures for the analysis of interactions occurring between enzymes and their ligands, both covalent and non-covalent binding (Benkestock et al., 2004). For example, mass spectrometry can be used as an alternative to traditional spectrophotometric methods for kinetic and mechanistic characterisation of enzymes, both by monitoring substrate consumption and product formation, but also by monitoring enzyme-substrate interactions (Sundqvist et al., 2007).
After the purification of AmpC β-lactamase from *E. cloacae* and the characterisation of the interactions with LY2183240 regioisomers, mass spectrometry experiments employing MALDI-TOF were performed in order to assess potential complex formation between the enzyme and regioisomers. Avibactam was used for comparison and validation purposes.
The mass spectra of the peaks of the cephalosporinase are depicted in Figure 5.9. The molecular masses of the AmpC β-lactamase and enzyme plus avibactam measured by MALDI-TOF were 39,134 and 39,399 Da, respectively. This represents a mass increase of 265 Da, which corresponds with the molecular mass of avibactam, suggesting that this inhibitor was covalently attached in a complex.
These findings are in good agreement with Stachyra and coworkers (2010). The authors demonstrated a similar mass spectrum after the production of a covalent complex between avibactam and two different β-lactamases. On this occasion, they used the AmpC β-lactamase from *E. cloacae* P99, exhibiting molecular masses of 39,237 and 39,502 Da for the enzyme P99 alone and together with avibactam, respectively.

On the other hand, a similar profile to avibactam was not observed for the regioisomers of LY2183240. Analyses revealed that the molecular mass of the enzyme showed no significant increase in the presence of the 1,5- or 2,5-isomer (Figure 5.10).

However, slight variations in the molecular mass of the enzyme during the analyses were noted. In the 1,5-isomer spectrum, for instance, the molecular mass of the enzyme was 39,111 Da, 23 Da less than the cephalosporinase alone (Figure 5.10A). In the case of the 2,5-isomer, a molecular mass of 39,141 Da was observed, 8 Da more than the enzyme alone.

These minor differences can be attributed to standard analytical variations of the technique employed. Typically, there is considerable variability in the baseline, noise level, and peak intensities in an assortment of MALDI spectra produced from the same sample. Furthermore, variations in ion current are noticed with consecutive laser shots fired at the same position on the target protein surface (shot-to-shot reproducibility), across different locations on the enzyme surface (region-to-region reproducibility) and between identical loadings of the same sample onto different targets (sample-to-sample reproducibility) (Duncan et al., 2008).

Nevertheless, an additional peak in both mass spectra of approximately 200 Da was observed. This extra peak correlates with sinapinic acid, which is frequently used as matrix for MALDI-TOF analyses, especially for peptides and proteins greater than 10 kDa in mass (Meetani & Voorhees, 2005).

These findings indicate that none of the regioisomers gave rise to a covalent bond with the catalytic residues of the β-lactamase, under the same conditions of avibactam. The detection of non-covalent complexes is difficult and intricate when MALDI-TOF is applied, primarily due to the classic matrixes, e.g., sinapinic acid, and frequently used organic solvents such as acetonitrile that tend to disrupt weakly bound complexes under laser irradiation (Farmer & Caprioli, 1998; Chiang et al., 2010).

The results together with findings in above sections suggest that these compounds act as competitive non-covalent inhibitors. In addition, these data correlate favorably well with the *in silico* study and further support the type of interaction with the enzyme.

Although the mass spectrometry analysis suggests that both regioisomers of LY2183240 have potential non-covalent interactions with the enzyme, as

demonstrated in the Section 4.3.4.6, it was not possible to determine the reversibility of the compounds tested, including the reference avibactam. The progressive inactivation of the cephalosporinase by both isomers revealed to be non-reversible after 18 hours of incubation. Generally, enzyme inhibitors can be classified as reversible or irreversible. Reversible inhibitors are those that bind to a protein in a way that the enzyme activity may be reconditioned. Usually, an equilibrium can be established between the enzyme and a reversible, non-covalent inhibitor (Bush, 1988). On the other hand, irreversible inhibitors can be more efficient than reversible inhibitors due to the ultimate permanent inactivation of enzymatic activity. This type of inhibition is the effect of a sequence of reactions, including a reversible complex that can dissociate to free enzyme and inhibitor or can progress to form a covalent complex (inactivated enzyme) (Bush, 1988; Drawz et al., 2010). Nonetheless, according to recent reports, there is one exception to these rules. Avibactam presents an unusual mechanism of inhibition, whilst it seems to act as a reversible inhibitor, it also has a covalent interaction with residue Ser^{64} in the catalytic site of AmpC β-lactamases (Ehmann et al., 2012; Lahiri et al., 2014).

As the data revealed non-covalent interactions together with non-reversible inhibitory activity (Section 4.3.4.6), these findings may indicate that both regioisomers have high affinities towards the cephalosporinase and dissociate very slowly from the enzyme.

This study has gone some way towards enhancing the understanding of the mode of action of LY2183240 regioisomers towards class C β-lactamases. Nevertheless, further experimental investigations are needed to determine the precise mechanism of inhibition mediated by these compounds.

With this in mind, analysis of crystal structures of the enzyme-bound ligands are fundamental in order to confirm the proposed binding mode and provide an accurate description of the conformation of the LY2183240 regioisomers towards the class C cephalosporinase.

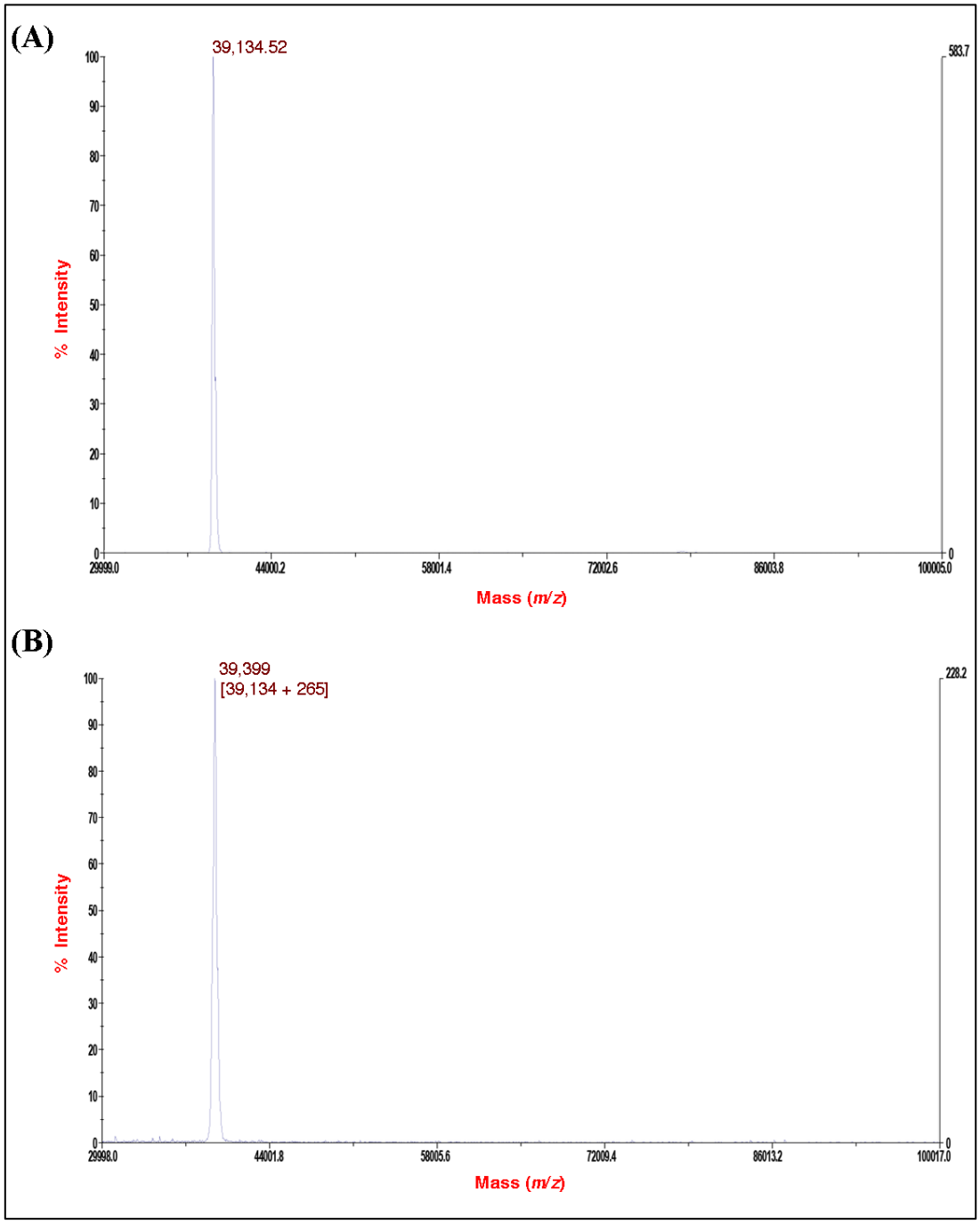


Figure 5.9. Mass spectra of β-lactamase from *E. cloacae* sp. **(A)** and inhibited by avibactam **(B)**.

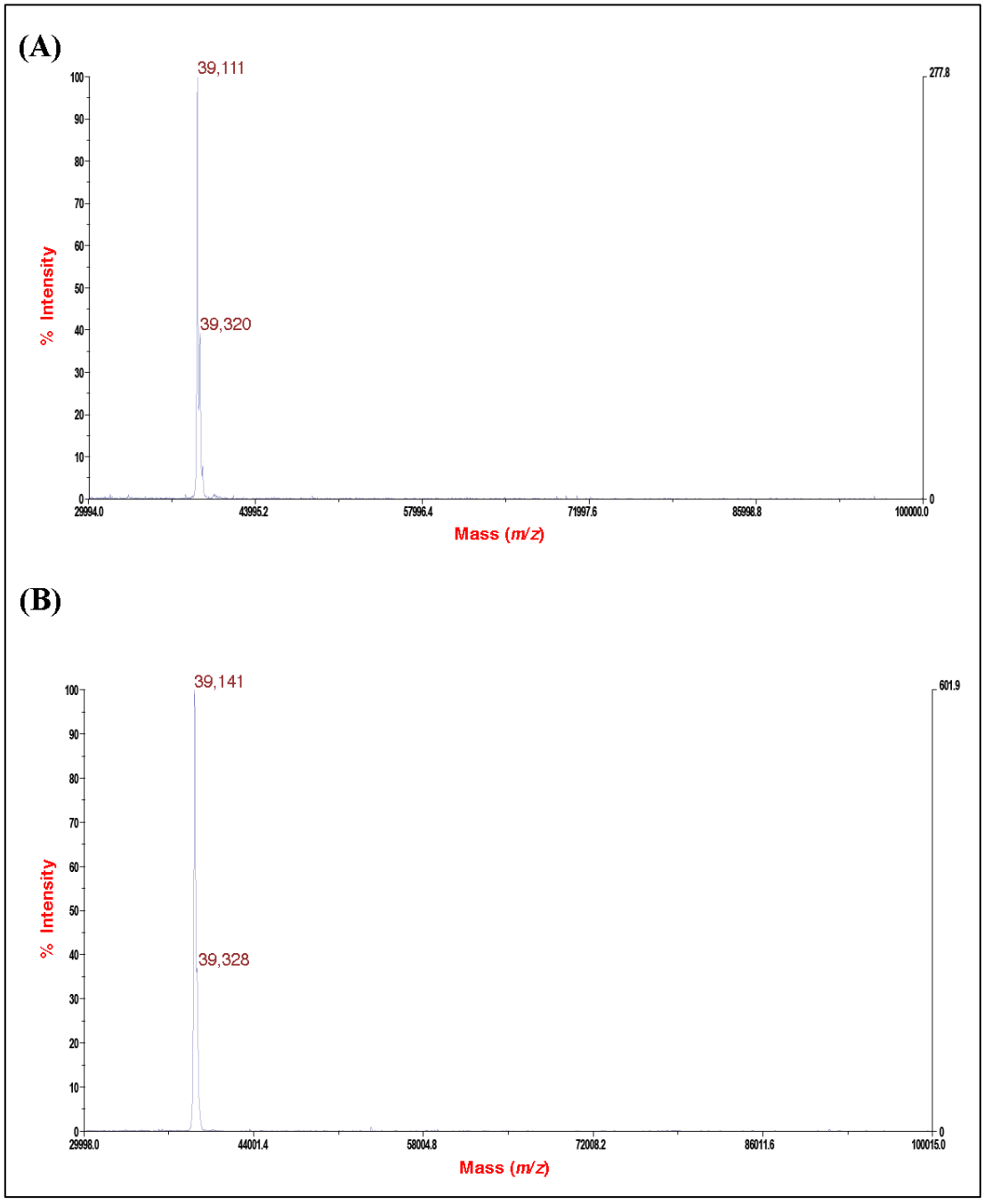


Figure 5.10. Mass spectra of β-lactamase from *E. cloacae* sp. inhibited by 1,5-LY2183240 **(A)** and inhibited by 2,5-LY2183240 **(B)**.

5.3.3 Crystallisation of AmpC with Avibactam and LY2183240 Regioisomers

The use of the three-dimensional structure of biological macromolecules in order to understand how they function is one of the most important fields of modern biology. To this end, the availability of atomic resolution structures offers a deep and unique understanding of protein function, and helps to discover the inner workings of the

living cell (Dessau & Modis, 2011). Furthermore, X-ray crystallography permits details of covalent and non-covalent interactions to be analyzed quantitatively in three dimensions, consequently providing the basis for the comprehension of binding of ligands to proteins as well as modes of action such as cell-surface binding (Palmer & Niwa, 2003).

Acquiring appropriate single crystals is the least comprehended step in the X-ray structural analysis of a protein. The science of protein crystallisation is still an underdeveloped area, although interest is growing, encouraged especially by microgravity experiments in space flights (Drenth, 1994).

Protein crystallisation is mainly a trial-and-error procedure in which the protein is slowly precipitated from its solution. The presence of impurities, crystallization nuclei, and other unknown factors play a role in this process (Drenth, 1994; Kundrot, 2004).

Growth of high quality single crystals is the basis of X-ray structure determination and also a limiting step. Protein purity and homogeneity are essential for the growth of single protein crystals. Crystallisation of macromolecules is a multi-parametric process involving three main steps: nucleation, growth and cessation of growth.

Nucleation, the initial process that occurs in the formation of a crystal from a solution, in which a small number of ions, atoms, or molecules become arranged in a pattern characteristic of a crystalline solid, forming a site upon which additional particles are deposited as the crystal grows (Uhlmann, 1980; Kashchiev, 2000).

The cessation of crystal growth can occur for a number of reasons. The most usual one is the decrease in concentration of the crystallising solute to the point where the solid and solution phases reach exchange equilibrium. In this case, the addition of more solute can result in continued crystal growth. Nevertheless, some crystals reach a certain size beyond which growth does not proceed, regardless of solute concentration. This may be the result of either cumulative lattice strain effects or poisoning of the growth surface (Thaller et al., 1981; Sato et al., 1992).

There are several techniques to crystallise proteins; all of them aim at bringing the solution of the protein to a super-saturation state, which will force the macromolecules into the solid state, the crystal.

Among the crystallisation micro-methods, vapor diffusion techniques are the most widely used (Benvenuti & Mangani, 2007). A micro-drop of sitting or hanging protein solution is mixed with buffer, crystallising or precipitant agent, and

eventually also additives, and equilibrated against a reservoir containing a solution of precipitant agent at a higher concentration than the droplet (Bunko et al., 2007).

The Figure 5.11 illustrates one of the many 24-well plates used for the vapor diffusion method screening, employing AmpC protein and inhibitor mixtures.

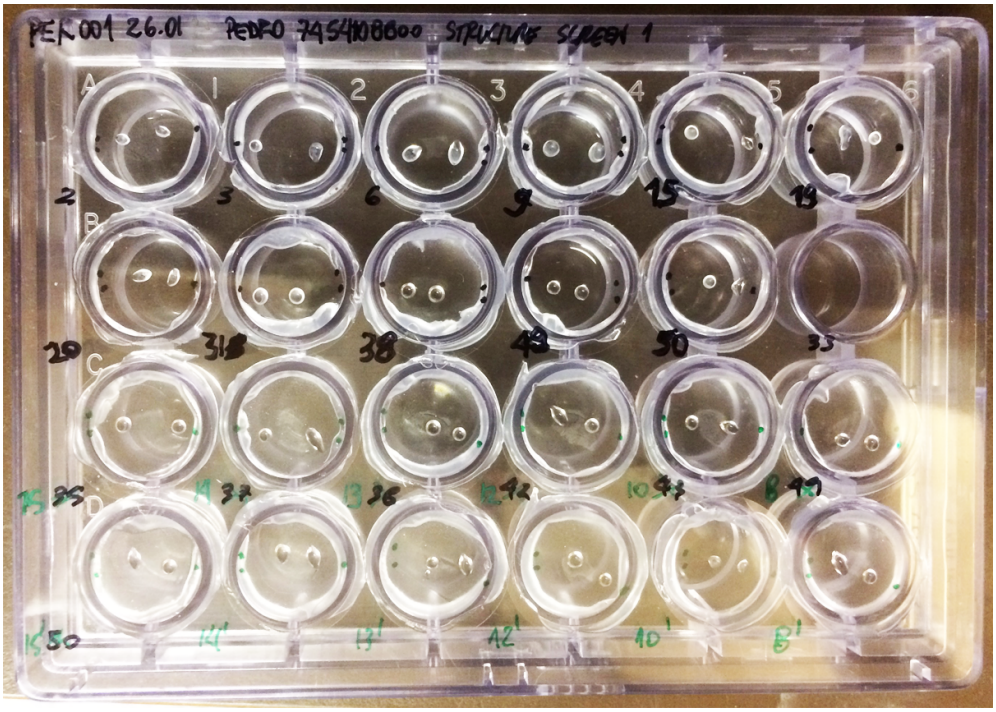


Figure 5.11. Screening for protein crystallization using vapor diffusion method.

There are many reports regarding the conditions of the crystallization of AmpC β-lactamase from different strains of Enterobacteriaceae species (Lobkovsky et al., 1993; Knox, 1995; Powers et al., 2002; Nukaga, Kumar, et al., 2003; Nukaga, Abe, et al., 2003; Yamaguchi et al., 2009; Lahiri et al., 2013; Lahiri et al., 2014).

In this current study, several attempts were made to achieve the best conditions to grow the crystals of the protein, and most importantly, the complex enzyme and inhibitors.

Table 20 depicts all the conditions undertaken using AmpC, LY2183240 regioisomers and avibactam.

The experiments varied in protein/inhibitor concentration ratio, precipitant agents, pH and temperature.

Table 20. Crystallisation screening conditions.

Precipitant Agents	Protein/Inhibitor Ratio	pH	Temperature (°C)
10 - 50% PEG 8,000	1:1 and 3:2	4.5 - 8	4 - 18
0.05 - 0.1M KH_2PO_4	1:1 and 3:2	4.5 - 8	4 - 18
0.05 - 0.1M K_2HPO_4	1:1 and 3:2	4.5 - 8	4 - 18
50 mM $(CH_3)_2AsO_2H$	1:1 and 3:2	4.5 - 8	4 - 18

The best conditions established for the co-crystallisation was employed; 20 % PEG 8,000 and 0.1-M KH_2PO_4 as precipitant agents at pH 5.0 and 17°C, and with a protein/inhibitor ratio of 3:2, as previously reported by Crichlow et al. (1999). The crystals appeared after approximately 20 days of incubation (Figure 5.12).

Surprisingly, the crystals only emerged in solutions containing inhibitors, in other words, the AmpC alone with the precipitant agents showed no growth, indicating that the presence of inhibitors influenced in the development of the crystals.

Figure 5.12A shows the crystals of AmpC in complex with the reference standard avibactam. The crystals presented with the shape of thin clustered needles, showing a similar form of a star. Preliminary X-ray data collection revealed that the size of the crystals were comparable to a protein (data not shown). However, due to the size of the crystals grown, it was not possible to continue the analysis and determine the protein-inhibitor complex structure. In this context, a microseeding technique was applied, which is a recognized method of obtaining crystal and is used as an optimization step, where seed crystals are transferred into conditions that are similar to those previously known to promote crystallisation (Stura & Wilson, 1991; Bergfors, 2003; Shaw Stewart et al., 2011). Unfortunately, after several endeavors to microseed and create more nuclei in the solution, no suitable crystals have been grown so far. One of the possible explanations was the small amount of protein used when compared with former studies. Crichlow and coworkers (1999) for instance, utilized 13.4 mg of AmpC from *E. cloacae* P99. Due to the long and laborious process of AmpC purification, a limited quantity of enzyme was obtained and employed in this crystallography study, making it difficult to reproduce the same conditions. Nevertheless, the study is still ongoing.

Figure 5.12B and 5.12C depict the crystals of AmpC together with 1,5-LY and 2,5-LY, respectively. Similar to the avibactam, both sets of crystals exhibited a thin needle shape, although much longer. Preliminary X-ray analysis revealed that the

crystals grown were very small for a protein, strongly suggesting that they are crystals of only the ligand, and not in complex with the enzyme. Curiously, the crystals of the LY2183240 compounds had grown only in the presence of the AmpC enzyme, and not alone as shown in the Figures 3.40D and 3.40E. In these pictures it was possible to see potential precipitates, however no crystals were observed under the same conditions.

These findings suggest that the presence of the AmpC helps somehow the growth of crystals of LY2183240 regioisomers, however more studies are necessary to comprehend better the circumstances of this phenomenon.

The microseeding technique was again applied for both LY2183240 compounds, however using crystals of the enzyme-avibactam complex (Figure 5.12A).

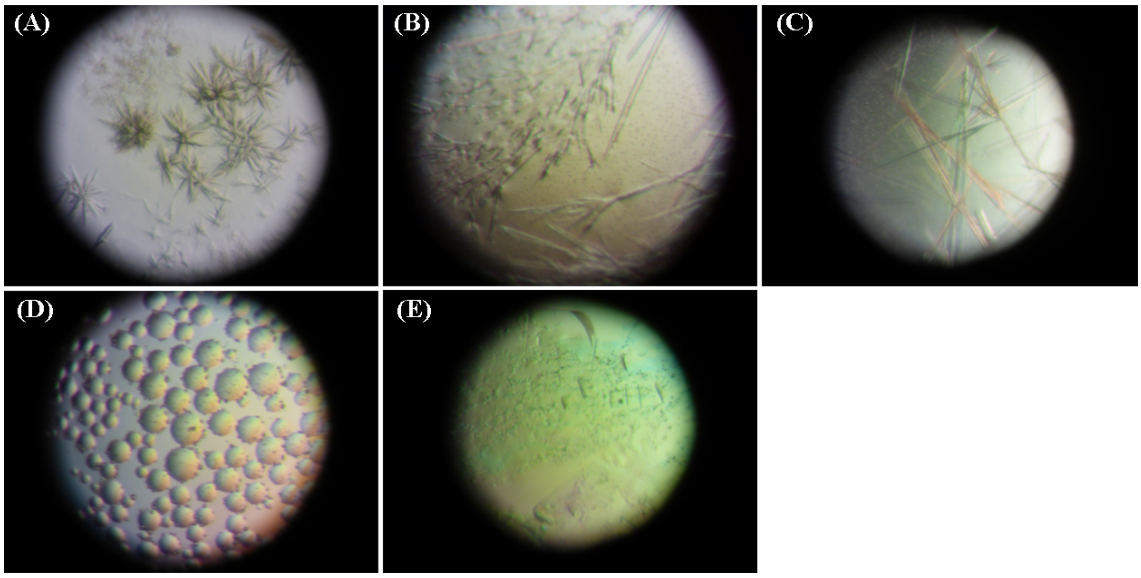


Figure 5.12 Crystals of AmpC protein from *E. cloacae*. (A) AmpC with avibactam, (B) AmpC with 1,5-LY2183240, (C) AmpC with 2,5-LY2183240, (D) 1,5-LY2183240 alone, (E) 2,5-LY2183240 alone.

Basically, the crystals were transferred to a solution containing AmpC, precipitant agents and the regioisomers, separately. The main objective was to create multiple nuclei and increase the chance of growing crystals of the protein-inhibitor complex. However, similarly to avibactam, the samples showed no crystals after 25 days incubation at 17°C.

Growing crystals is not an easy task. Not only do crystals need be grown, but they also must be of high quality, and suitable for high-resolution X-ray diffraction

analysis (McPherson, 1990). The reason that the crystallisation step turned into the primary obstacle to increase structural knowledge is the empirical nature of the methods employed to overcome it (McPherson, 1982). As mentioned before, macromolecules, such as proteins, are extremely complex physical-chemical systems whose properties vary as a function of many environmental influences, including temperature, pH, ionic strength, contaminants and solvent composition, among others. Proteins, in general, are structurally dynamic, heterogeneous and aggregating systems, and they can change conformation in the presence of ligands (Schulz & Schirmer, 1979; Creighton, 1993). Protein crystallisation is, hence, a matter of searching, as systematically as possible, the ranges of the individual parameters that impact upon crystal formation, finding a set or multiple sets of these factors that yield some kind of crystals, and then optimizing the variable sets to obtain the best possible crystals for X-ray analysis. This can be achieved conducting a long series, or establishing a vast range, of crystallisation trials, evaluating the results, and using information acquired to improve matters in successive rounds of trials. Since the number of variables is so large, and their ranges so extensive, intelligence and intuition in designing and evaluating the individual and collective trials becomes essential (McPherson, 1990).

It was possible to establish the best conditions and success in generating preliminary AmpC crystals was achieved. However the limited availability of purified enzyme after multiple trials to find the best crystallization conditions at the time meant the optimum protein quantity could not be used to reproduce previous studies (Crichlow et al., 1999; Lobkovsky et al., 1993; Lahiri et al., 2014). The crystallisation study is still in progress.

5.4 Conclusions

The molecular modelling studies corroborate earlier observations suggesting that LY2183240 regioisomers bind within the catalytic site of class C β-lactamases, interacting with some of the same residues, including Tyr^{150}, Lys^{315} and Thr^{316}, used by the substrate nitrocefin and standard β-lactamase inhibitors. Also, these results substantiate the competitive inhibition model previously determined by enzyme kinetic studies on Chapter 4. Furthermore, mass spectrometry data revealed non-covalent interactions between AmpC β-lactamase and LY2183240 regioisomers, which together with the non-reversible inhibitory activity, may indicate that both regioisomers present a high affinity towards this cephalosporinase and dissociate very slowly from the enzyme.

Although it was possible to grow crystals of AmpC enzyme, the size and quality of them meant they were not suitable for a high resolution X-ray diffraction analysis. Currently, the crystallography study is still in progress and results are being obtained.

These findings have considerable biological implications once it is largely recognized that β-lactamase enzymes are the most widespread resistance mechanisms to β-lactam antibiotics. Clinically used inhibitors for these enzymes resemble substrates; both contain a β-lactam ring. For this reason, resistance develops rapidly because mechanisms that depend on recognition of the lactam ring already exist. A novel non-β-lactam inhibitor may evade such mechanisms, and, most importantly, it would not be hydrolyzed by β-lactamases.

6 CHAPTER 6

Concluding Remarks and Future Perspectives

The increase and spread of antimicrobial resistance presents a paramount challenge to both science and medicine. This global crisis reflects the worldwide misuse and overuse of these drugs and the lack of development of new antibiotic agents by the pharmaceutical industry to address the challenge (Brown & Wright, 2016). Amongst the many antibiotic resistance mechanisms towards β-lactams, the production of β-lactamases by bacteria remains one of the most significant threats to the efficacy of this life-saving class of antimicrobial agents (Drawz et al., 2014). Multi-drug resistant organisms, such as methicillin-resistant *S. aureus* (MRSA), are also a serious global problem; alarming reports from different parts of the world indicate a threat to the healthcare system and individual patients (Andersson et al., 2011). More recently, evidence that MRSA is becoming resistant to glycopeptides, such as vancomycin, and newer therapies raises concern about the use of these therapies in clinical practice (Stryjewski & Corey, 2014). Consequently, there is a need to develop new antimicrobial agents and resistance-modifying agents such as β-lactamase inhibitors.

No previous studies on the antimicrobial or resistance-modifying features of LY2183240 regioisomers have been reported in the literature, indeed this is the first report on the activity of the compound towards prokaryotic systems. This study provided an exciting opportunity to advance the knowledge of a potential and novel anti-staphylococcal agent with β-lactamase inhibitory properties.

As such, LY2183240 could be useful as a novel scaffold in the design of future antimicrobial drugs as well as inhibitors with improved activities towards class C β-lactamases (and possibly other enzymes).

The crystallography study made in collaboration with Dr Gary Parkinson (UCL School of Pharmacy) is a work in progress, once the co-crystallization of the cephalosporinase and the LY2183240 regioisomers has been achieved insights into the binding of the compounds can be made assisting mechanistic elucidation.

Moreover, future work would also focus on kinetics studies using purified FabI and 2,5-LY2183240 in order to confirm if this enzyme is indeed the target responsible for the antimicrobial activity of the compound. Alternatively, a radioligand binding experiment could be performed to identify the target or targets for the anti-staphylococcal effect. However, the regioisomers of LY2183240 may not have suitable groups to add a radioligand. Moore and coworkers (2005), for instance, created a radioligand and photoaffinity probe, adding iodo and azido groups to LY2183240. However, this alteration in the structure resulted in a modest shift of uptake inhibition

potency. In this sense, determination of the primary target responsible for the anti-staphylococcal activity may be complex as illustrated by this study.

Another important aspect for future investigation is the synthesis and production of analogues of LY2183240 in order to assess and improve the potency of the activities already observed. Ortar and coworkers (2007) synthesized a series of 1,5- and 2,5-disubstituted carbamoyl tetrazoles, evaluating the activity towards anandamide, FAAH and other relevant targets. The study demonstrated that changes in the structure could improve the potency and selectivity of the effect. Consequently, this is an interesting approach for enhancing the antimicrobial and β-lactamase inhibitory properties of the molecule.

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8 APPENDIX

8.1 NMR Analyses

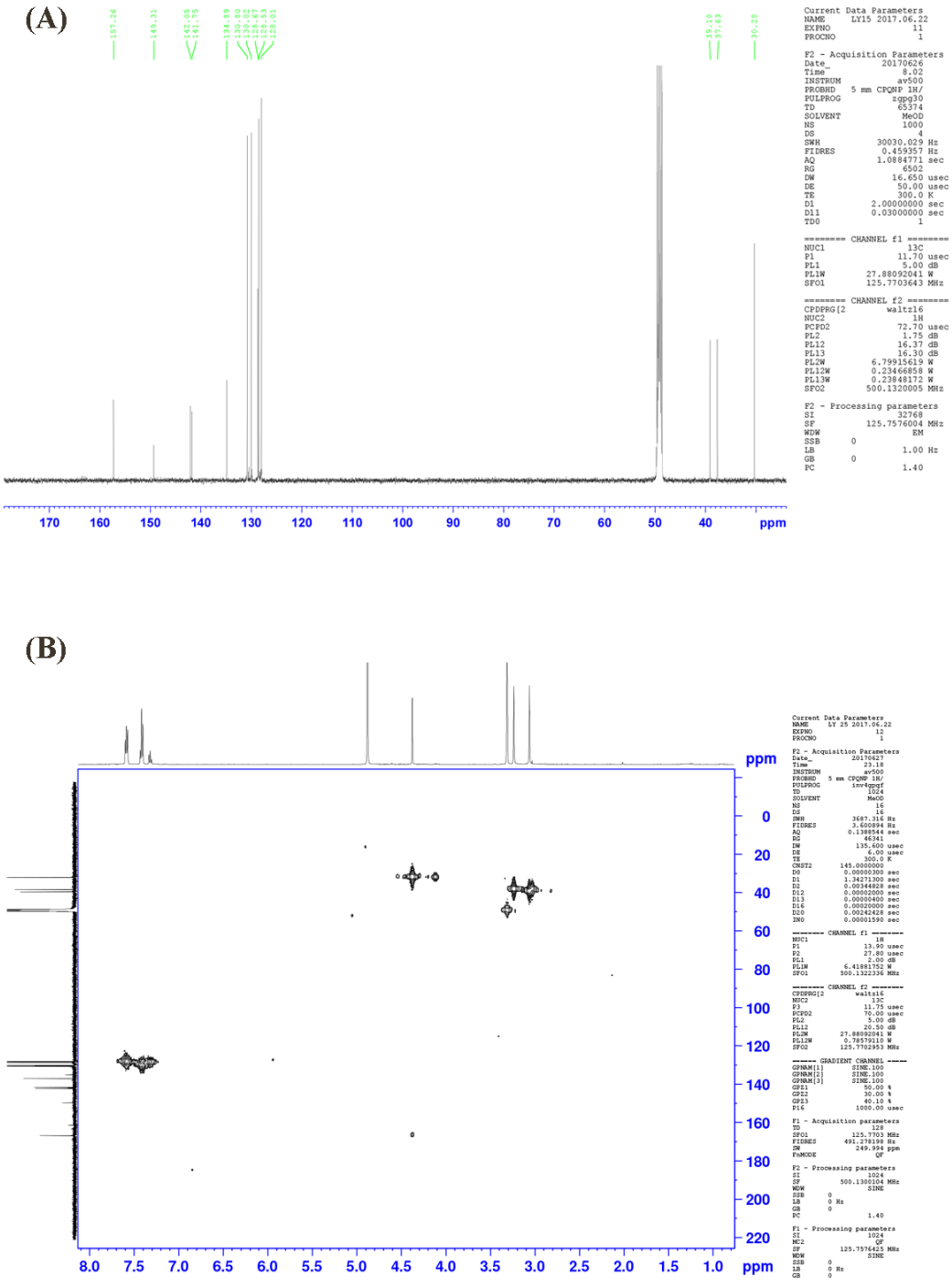

Figure 8.1. (A) ^{13}C and **(B)** HMQC NMR spectra of 1,5-LY2183240 recorded in MeOH-d4.

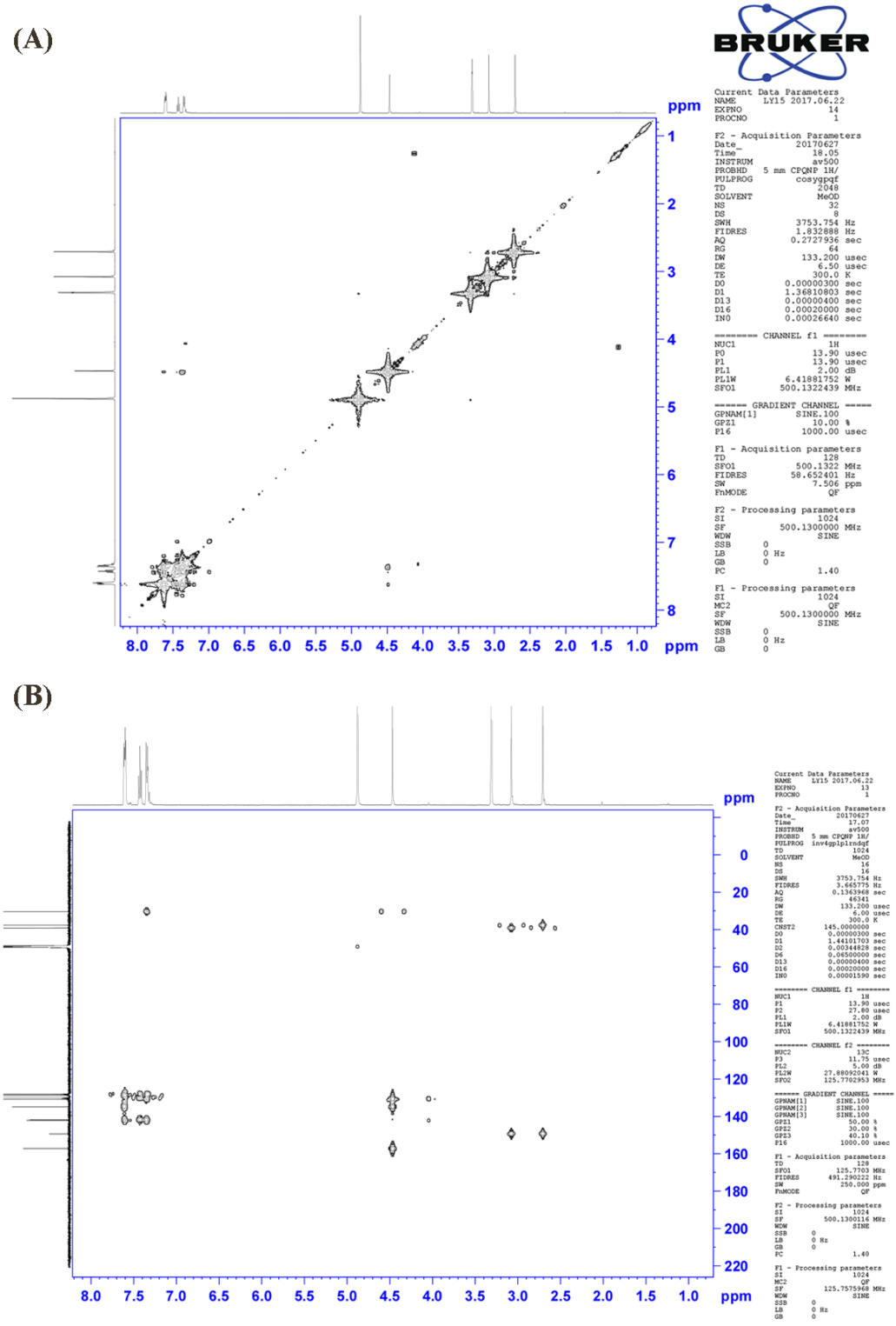

Figure 8.2. (A) COSY and (B) HMBC NMR spectra of 1,5-LY2183240 recorded in MeOH-d4.

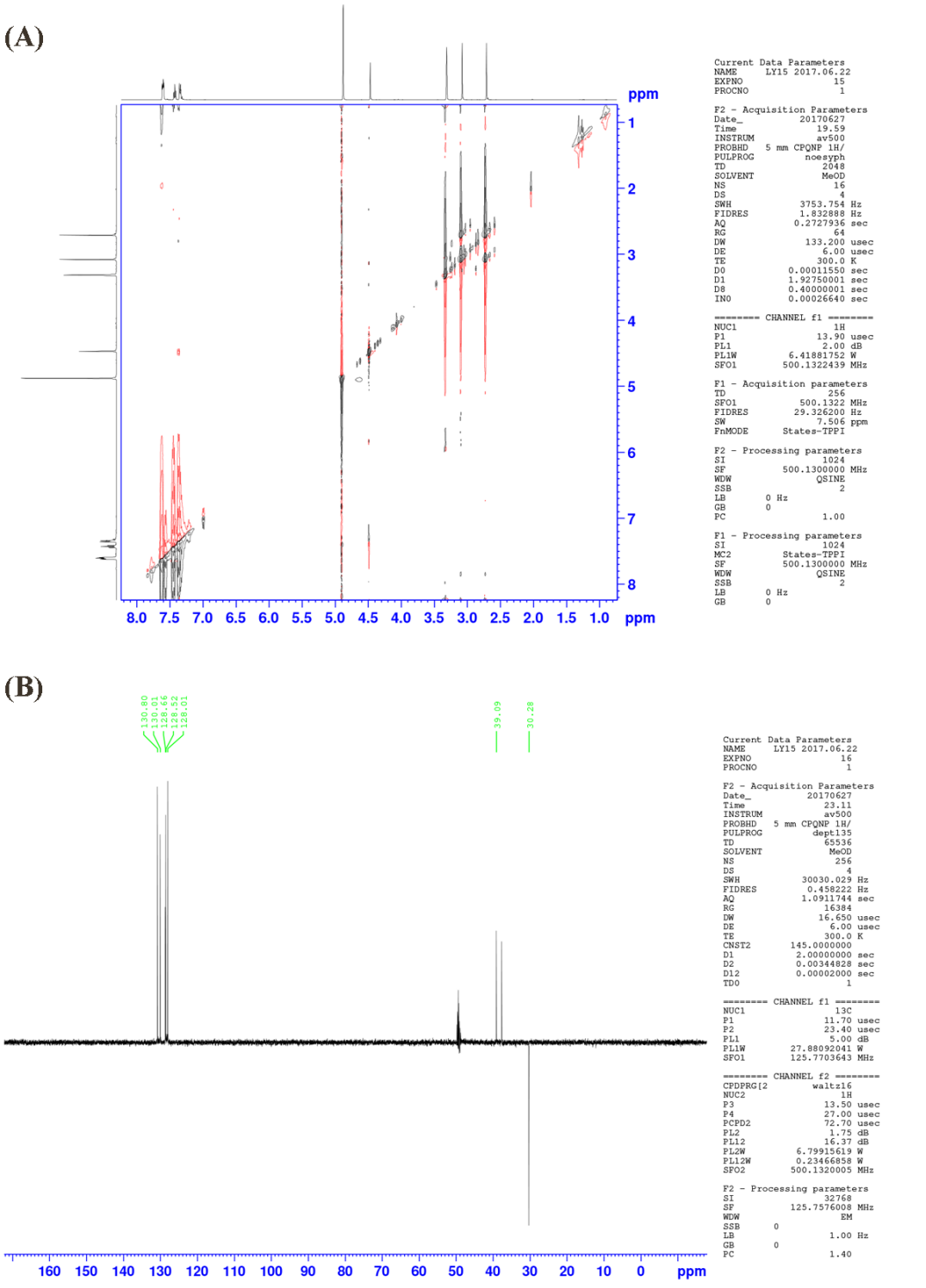

Figure 8.3. (A) NOESY and **(B)** DEPT NMR spectra of 1,5-LY2183240 recorded in MeOH-d4.

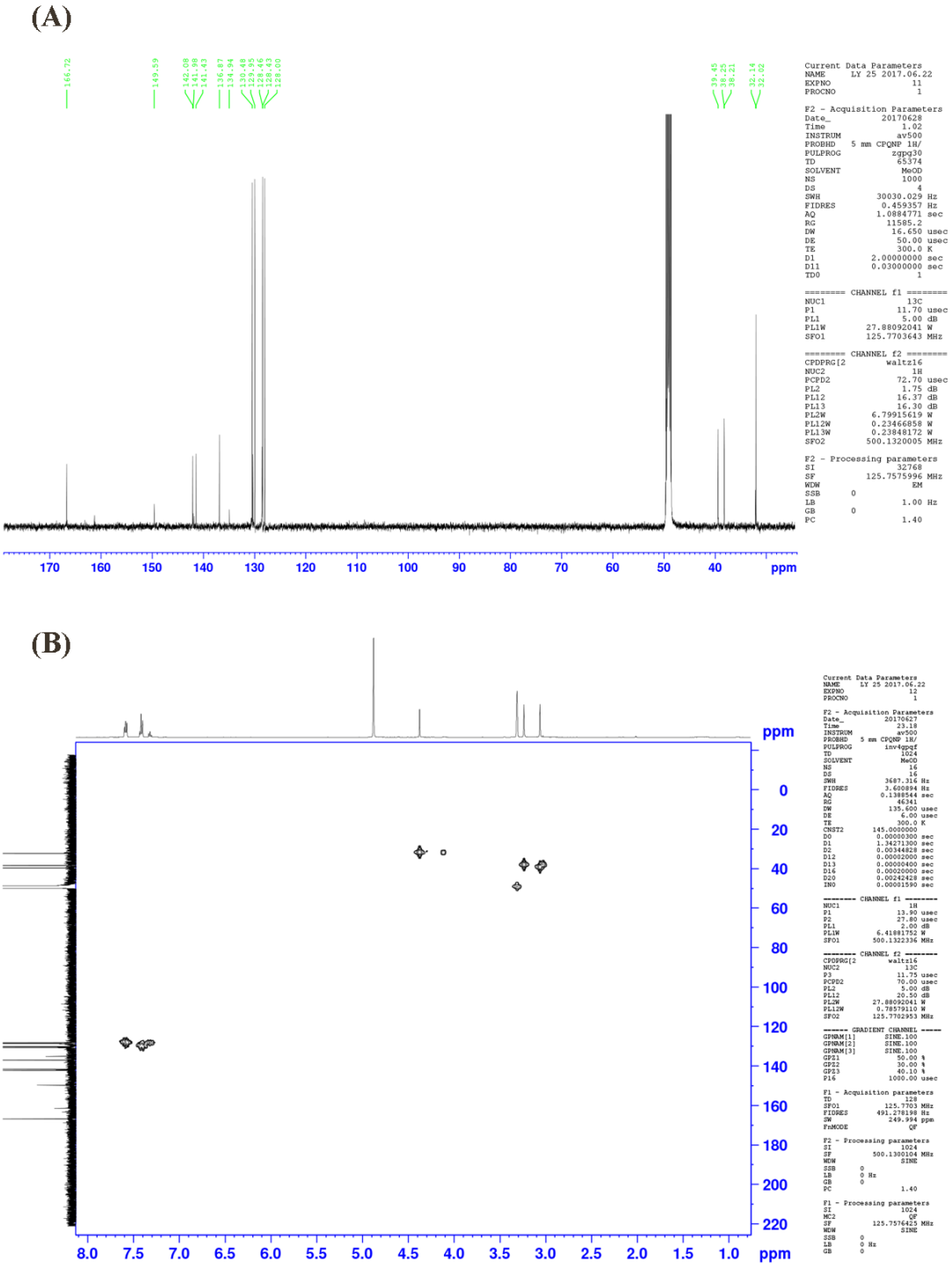

Figure 8.4. (A) ^{13}C and **(B)** HMQC NMR spectra of 2,5-LY2183240 recorded in MeOH-d4.

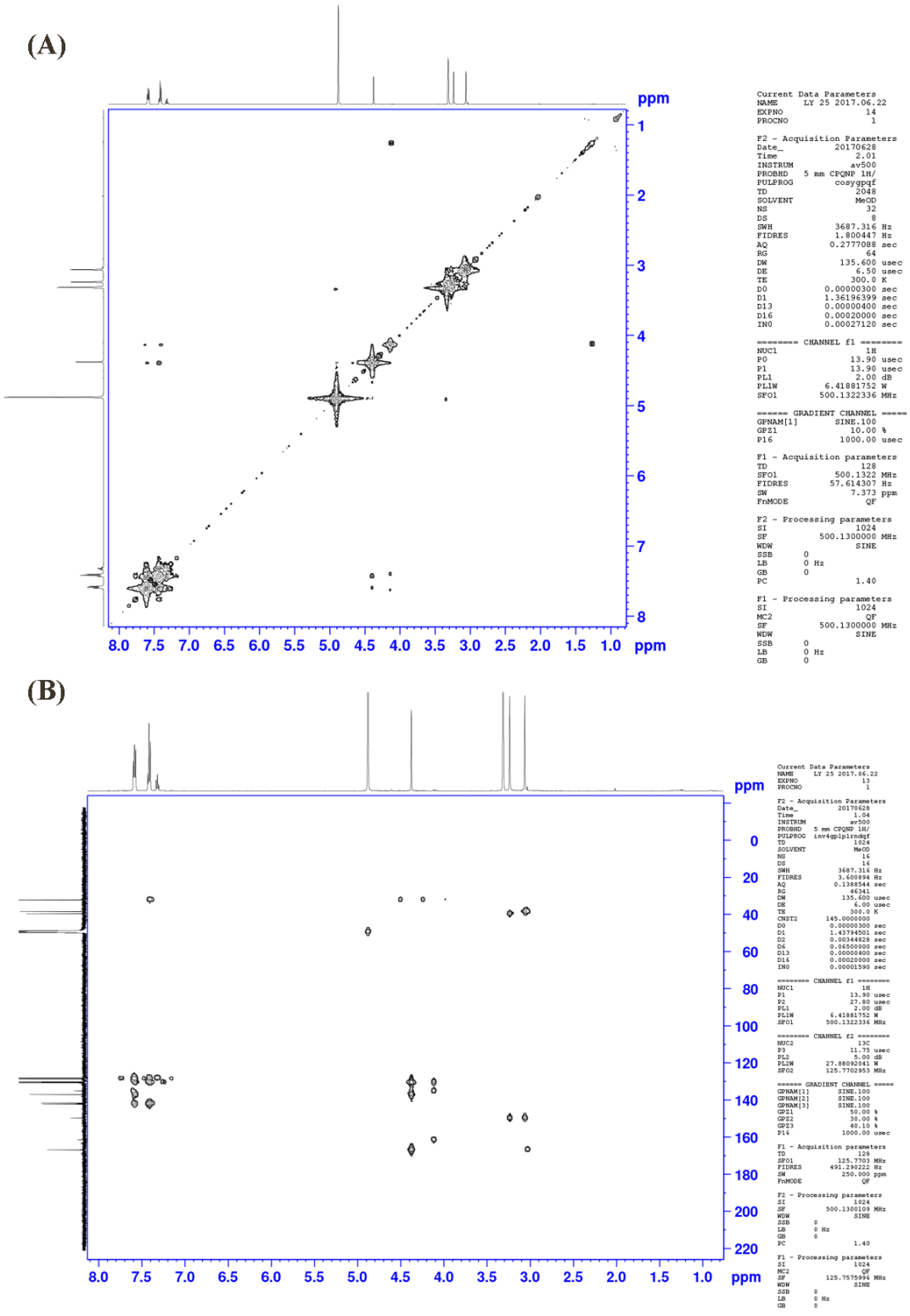

Figure 8.5. (A) COSY and **(B)** HMBC NMR spectra of 2,5-LY2183240 recorded in MeOH-d4.

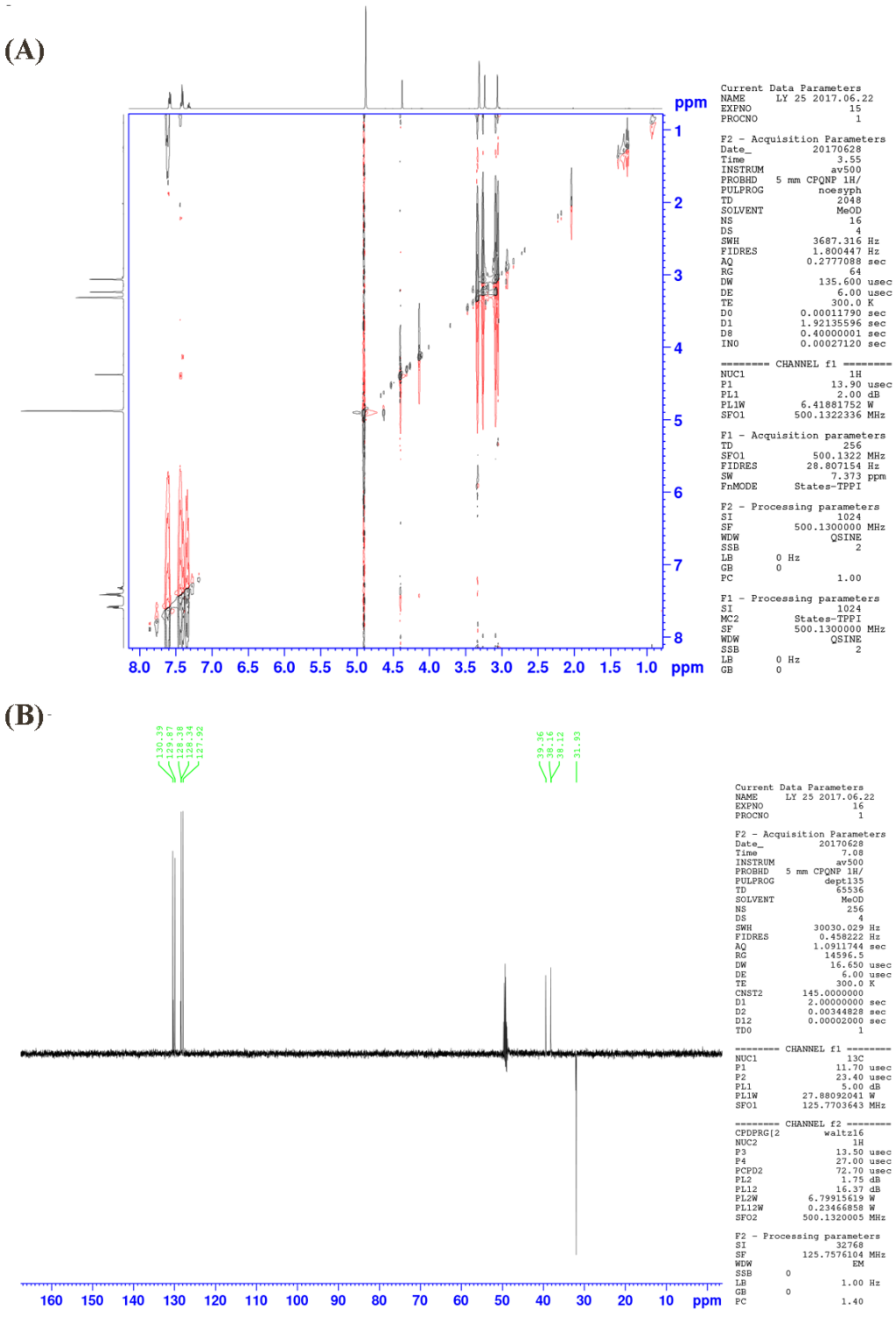

Figure 8.6. (A) NOESY and **(B)** DEPT NMR spectra of 2,5-LY2183240 recorded in MeOH-d4.

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